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ISOLATION AND CHARACTERIZATION OF MUTANTS OF SACCHAROMYCES CEREVISIAE CONDITIONALLY DEFICIENT IN EITHER NUCLEAR OR MITOCHONDRIAL DNA METABOLISM.

CITY UNIVERSITY OF NEW YORK, PH.D., 1978

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ISOLATION AND CHARACTERIZATION OF MUTANTS OF *SACCHAROMYCES CEREVISIAE* CONDITIONALLY DEFICIENT IN EITHER NUCLEAR OR MITOCHONDRIAL DNA METABOLISM

by

BERISH RUBIN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1978

Berish Rubin

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

In this communication the isolation and characterization of single nuclear gene mutants of *Saccharomyces cerevisiae* conditionally deficient in DNA metabolism is reported.

The first mutant described in this communication has been found to be conditionally deficient in mitochondrial DNA metabolism. Growth of this strain at the restrictive temperature of 36°C in dextrose or galactose containing media results in the induction of neutral petites which have been found to be devoid of mitochondrial DNA. Growth of this strain at the restrictive temperature in glycerol containing media results in the induction of suppressive petites that contain mitochondrial DNA. Analysis of the rate of incorporation of radioactive adenine into the mitochondrial DNA of this strain at the restrictive temperature in either dextrose, galactose, or glycerol containing media demonstrates that this rate is greatly reduced. This reduced rate of incorporation appears to involve the synthetic aspects of mitochondrial DNA metabolism, as cells prelabeled with ^{3}H-adenine show no loss or degradation of mitochondrial DNA at the restrictive temperature.

The second mutant described in this communication has been found to be conditionally deficient in nuclear DNA metabolism. Growth of this mutant strain at the restrictive temperature of 36°C results in a rapid loss of cell viability while the optical density of the

experimental culture continues to increase. No apparent reduction in the rate of isotope incorporation into DNA was detected during this period. The loss of viability expressed in this strain appears to require the continued growth of the mutant cells, since cells that have been starved prior to and during exposure to the restrictive temperature fail to exhibit any loss of cell viability even after seven hours. When haploid cells carrying this temperature sensitive lesion were exposed to the restrictive temperature for varying lengths of time, returned to the permissive temperature, mated with a non-temperature sensitive strain and then the resulting diploids made to undergo meiosis, a greatly reduced number of viable spores were produced. Genetic analysis of the viable spores obtained from these diploids has revealed aberrant auxotrophic marker segregation patterns which appear to be the result of aneuploidy in these meiotic products.

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INTRODUCTION

The first study reported in this communication, in which a temperature sensitive mutant of *Saccharomyces cerevisiae* defective in mitochondrial DNA metabolism is described, was undertaken to determine if there are any nuclear genes which are involved exclusively in the synthesis of mitochondrial DNA. Our selection for such mutants involved the isolation of strains which, at the restrictive temperature, induced petites that were neutral and were unable to synthesize mitochondrial DNA at the permissive temperature. Our reasoning behind this mode of selection was that if the petites induced were both neutral and unable to synthesize mitochondrial DNA, the conditional mutation must affect either the synthesis or the maintenance of the mitochondrial DNA. The isolation and characterization of such a mutant is described herein.

Following the discovery in 1963 of a species of mitochondrial DNA in mammalian cells by means of cytochemical and electron microscopic studies (Nass and Nass, 1963), there has been a great deal of interest in the biogenesis of this cytoplasmic organelle.

Mitochondrial DNA

In 1964 the unequivocal identification of mitochondrial DNA from *Neurospora* by means of cesium chloride density gradient centrifugation was reported by Luck and Reich (1964). In their analysis they demonstrated that the DNA species with the bouyant density of 1.701 gm/cm^3, which was ordinarily seen as a shoulder on the nuclear DNA (1.712 gm/cm^3)

could be enriched for by extracting DNA from isolated DNase treated mitochondria. Subsequently the mitochondrial DNAs from numerous sources have been characterized. Generally, in animals, the molecule is circular with a circumference of about 5 to 6 μm (corresponding to a molecular weight of approximately 1×10^7 daltons), while in protists and plants the mitochondrial DNA appears to be much longer, 15 to 30 μm (corresponding to a molecular weight of 2.9 to 6×10^7 daltons), and is generally, but not always, circular (Borst, 1972). The circular DNAs isolated from the mitochondria of a variety of organisms have been found to exhibit superhelical turns or supertwists (Helinski and Clewell, 1971). In the yeast *Saccharomyces*, initial attemps to demonstrate the circularity of its mitochondrial DNA were unsuccessful and only yielded heterogeneous linear DNA molecules which were either equal to or less than 26 μm in length. Circular 25 μm mitochondrial DNA was eventually isolated by Hollenberg and coworkers (1970) after osmotically lysing the mitochondria of yeast.

The mitochondrial DNA of protists such as *Neurospora* (Luck and Reich, 1964) and yeast (Moustacchi and Williamson, 1966) have been found to have a base composition that is distinct from that of the nuclear DNA and can thus be separated from the nuclear DNA by means of cesium chloride density gradient centrifugation, hydroxyapatite chromatography (Bernardi *et al.*, 1972) and polylysine chromatography (Blamire *et al.*, 1972b). In many of the organisms that do not possess

a mitochondrial DNA whose buoyant density is distinct from that of the nuclear DNA, the presence of the mitochondrial DNA in a circular form can be taken advantage of. Such circular molecules can be separated from the linear DNAs by the use of dyes, such as ethidium bromide, which affects the buoyant density of circular DNA differently than linear DNA (Bauer and Vinograd, 1970).

Early studies directed at determining the mechanism of mitochondrial DNA replication revealed that this DNA was conserved in some fashion, however, semi-conservative replication could not be clearly established (Reich and Luck, 1966). Subsequent analysis has demonstrated semi-conservative replication of mitochondrial DNA in a variety of organisms (Gross and Rabinowitz, 1969; Cummings, 1977). The mode of mitochondrial DNA replication is still a matter of controversy. Williamson and Fennel in 1974 and Sena and coworkers in 1975 performed downshift experiments with ^{15}N labeled cells and found that the replication of mitochondrial DNA occurs dispersively. However, in a paper presented recently (Leff and Eccleshall, 1977) it was reported that the mitochondrial DNA of yeast replicates in a semi-conservative manner. In this communication using deoxybromouridylate the replication of the mitochondrial DNA was monitored for three generations and found to replicate in a semi-conservative non-dispersive manner. This apparent discrepancy may be due to the different rates of incorporation of these two density labels into the mitochondrial DNA or to

the different degrees of recombination occurring to this DNA in these experimental strains. In order to avoid the problems encountered with an in vivo system, Mattick and Hall in 1977, developed an *in vitro* system for mitochondrial DNA synthesis using isolated mitochondria from *Saccharomyces cerevisiae* in which extensive synthesis of mitochondrial DNA was observed. These investigators observed semi-conservative replication of the mitochondrial DNA.

The time in the cell cycle that mitochondrial DNA synthesis takes place appears to vary depending on the organism studied. Mitochondrial DNA has been found to be synthesized at a relatively constant rate during the entire cell cycle in *Physarum* (Braun and Evan, 1969) and *Tetrahymena* (Cameron, 1966). Mouse fibroblast mitochondrial DNA replicates throughout the cell cycle, although maximal replication appears to occur in early S phase (Madreiter *et al.*, 1972). Depending on the cell synchrony procedure, HeLa cells either replicate their mitochondrial DNA in S phase and G_2 or constantly during the entire cell cycle (Pica-Mattocia and Attardi, 1972). *Euglena gracilis* mitochondrial DNA has been found to be replicated during S phase of the cell cycle (Calavayrac *et al.*, 1972). Conflicting conclusions regarding the timing of mitochondrial DNA synthesis in *Saccharomyces cerevisiae* have been published. In 1970, on the basis of some preliminary data, Cottrell and Avers reported that in *Saccharomyces cerevisiae* mitochondrial DNA synthesis occurs synchronously before S phase. Williamson and Moustacchi in 1971, however, report-

ed continuous synthesis of mitochondrial DNA during mitosis. In a recent communication Sena and coworkers further substantiated the continual synthesis of mitochondrial DNA during the cell cycle. In this work in which DNA replication was monitored by ^{14}N incorporation into ^{15}N labeled DNA, it was demonstrated that unlike nuclear DNA which is synthesized discontinuously during the cell cycle, mitochondrial DNA is replicated continuously. This independence of the timing of mitochondrial DNA synthesis suggests that there exists unique replication control systems for these two DNA species. This suggestion has been confirmed in experiments in which it was found that treatment of cells with cycloheximide (Grossman _et al._, 1969), an inhibitor of cytoplasmic protein synthesis, or α factor (Petes and Fangman, 1973), a sexual pheromone, inhibits the synthesis of nuclear DNA but allows the mitochondrial DNA to continue to be synthesized. Furthermore, when the synthesis of mitochondrial DNA in several cell division cycle (cdc) mutants conditionally affecting either the initiation of nuclear DNA synthesis or nuclear division was monitored at the restrictive temperature, it was found (Newlon and Fangman, 1975) that the mitochondrial DNA continued to be synthesized long after nuclear DNA synthesis was halted. The mitochondrial DNA synthesis system is, however, not entirely independent of the nuclear system for it has been shown that genes affecting the elongation of nuclear DNA do in fact block the synthesis of the mitochondrial DNA species as well (Newlon and Fangman, 1975).

In 1972, Nagley and Linnane addressed themselves to the question as to whether the mitochondrial genome plays a role in the control of mitochondrial DNA synthesis. These investigators observed that the cellular levels of mitochondrial DNA in cells containing altered mitochondrial DNA was close to mitochondrial DNA levels in their normal wild type parents. This observation suggests that mitochondrial DNA synthesis is independent of any control by the mitochondrial genome. In a recent communication, Hall and coworkers (1976) reported a genetic analysis of the control of mitochondrial DNA levels and found that the level of this DNA in a cell is under the control of several nuclear genes.

Mitochondrial RNA

In 1968, Wintersberger and Viehauser reported the characterization of mitochondrial RNA isolated from highly purified mitochondria. This mitochondrial RNA, when subjected to sucrose gradient centrifugation, was found to separate into three major species with sedimentation values of 4S, 16S and 23S. These three RNA species were all found to hybridize preferentially with mitochondrial DNA. The 4s RNA was found to be transfer RNA, while the two faster sedimenting RNAs have subsequently been found to be ribosomal RNA (Fauman et al., 1969). Recent hybridization analysis has demonstrated that the mitochondrial DNA of yeast contains one cistron for each of the two mitochondrial RNAs (Borst and Grivell, 1971) as well as the information coding for 20-25 transfer RNAs (Martin et al., 1976).

In 1975, using poly U sepharose chromatography, Hendler and coworkers isolated poly A containing RNA from the mitochondria of yeast which was later shown to be messenger RNA. The isolated mitochondrial poly A containing RNA, whose synthesis has been found to be blocked with the addition of ethidium bromide (Hendler _et al._, 1975), is of a high molecular weight and exhibits a sucrose gradient profile similar to that obtained from HeLa cell poly A RNA (Hirsch and Penman, 1973). The messenger characteristic of this poly A containing RNA was demonstrated in an experiment in which this RNA was found to have the ability to direct, in an E. coli cell free system, the translation of the three peptides of the yeast cytochrome oxidases (Padmanaban _et al._, 1975) that have been shown to be synthesized by the yeast mitochondria in vivo (Mason and Schatz, 1973).

Mitochondrial Ribosomes

The isolation and characterization of mitochondrial ribosomes has been difficult to accomplish. Nevertheless, investigators have pursued the mitochondrial ribosome, spurred on by the presence of the transfer and ribosomal like RNAs isolated from mitochondria. In 1970, Morimoto and coworkers characterized the mitochondrial ribosome from yeast and demonstrated its activity in _in vitro_ amino acid incorporation. The mitochondrial ribosome of yeast have been found to sediment at about 73S and to be composed of a 50S and 38S subunit (Borst and Grivell, 1971). Mitochondrial ribosomes have been found

to resemble bacterial ribosomes in their sensitivity to low magnesium ions, chloramphenical, erythromycin, and to many other inhibitors of bacterial protein synthesis. The mitochondrial ribosome further resembles the bacterial ribosome in its resistance to cycloheximide, a classical inhibitor of protein synthesis on the cytoplasmic ribosome (Lamb *et al.*, 1968), and in its requirement for N-formylmethionine at the initiation of protein synthesis (Smith and Marcker, 1968).

Mitochondrial Protein Synthesis

The identification of the products of the mitochondrial protein synthesizing system is currently being investigated by means of biochemical and genetic analysis. The biochemical determinations usually involve either an analysis of the proteins present in the mitochondria of cells whose mitochondrial or cytoplasmic protein synthesis has been blocked by various antibiotics or an analysis of the proteins labeled in vivo with radioactive formate (see Schatz and Mason, 1974). The results thus far suggest that three of the seven polypeptides associated with the cytochrome oxidase (Mason and Schatz, 1973), four of the ten polypeptides of the oligomycin sensitive ATPase (Tzagoloff and Meagher, 1972), and three of the seven polypeptides associated with the cytochrome b complex (Marjanen and Ryrie, 1976) are made on the mitochondrial ribosome. The genetic identification of the mitochondrially coded products has involved the isolation of mutants of *Saccharomyces* which have retained mitochondrial protein synthesis but are unable to grow on glycerol

media. These mutants were analyzed and designated as being of a cytoplasmic nature if they fulfilled the following two criteria: (1) fail to be complemented by a ρ^o tester derived from a respiratory competent strain and (2) show non-Mendelian segregation patterns (4:0 and 0:4) of the mutations (Tzagoloff *et al.*, 1975a). A number of mutants satisfying these two criteria (termed mit) have been isolated and have been found to exhibit defects in the genetic loci coding for cytochrome oxidase (Slonimsky and Tzagoloff, 1976) mitochondrial ATPase (Tzagoloff *et al.*, 1975c) and the cytochrome b complex (Tzagoloff *et al.*, 1976). In this mutant isolation,nuclear mutants defective in the mitochondrial ATPase and respiratory enzymes were isolated as well (Tzagoloff *et al.*, 1975b).

Antibiotic Resistance

In addition to the existence on the mitochondrial DNA of information coding for components of various mitochondrial enzymes, numerous mitochondrial mutants have been isolated which exhibit increased resistance to (1) inhibitors of mitochondrial protein synthesis such as erythromycin, paromomycin, spiramycin, chloramphenical and mikamycin, and to inhibitors of (2) oxidative phosphorylation such as oligomycin, triethyltin and venturicidin. The isolation of such mutants has enabled an in depth genetic analysis of the yeast mitochondrial DNA (see Linnane *et al.*, 1974) as well as an analysis of the patterns of mitochondrial segregation (Wilkie and Thomas, 1973). The resistance expressed by many of the mitochon-

drial mutants to inhibitors of protein synthesis has recently been attributed to alterations of the mitochondrial rRNA (Faye et al., 1974), while the resistance to inhibitors of oxidative phosphorylation can usually be associated with alterations of the mitochondrially coded subunits of the ATPase (Tzagoloff et al., 1976).

Restriction Endonuclease Digestion

In the past few years, the use of restrictive endonucleases to specifically cleave DNA has aided in the identification and localization of various genes. Restrictive endonucleases make a limited number of duplex cleavages in DNA by recognizing specific nucleotide sequences and thus provide DNA fragments useful in physical mapping studies. Morimoto and coworkers, 1977, using the restriction endonucleases Eco RI, Hpa I, Bam HI, Hind III, Pst I and Sal I were able to create a physical map of the restriction sites on the yeast mitochondrial DNA. In this analysis, a comparison of mitochondrial DNA from four grande strains revealed similar, but slightly varying restriction patterns, with an identical genome size of approximately 5×10^7 daltons or 75 kilobases. Analysis of the restrictive patterns of a fifth grande strain revealed a different pattern from the above described strains and a genome size of 70 kilobases. The variability detected amongst these grande strains was further substantiated in a study (Prunell et al., 1977) in which a comparison of various different strains of *Saccharomyces cerevisiae* revealed large differences in electrophoretic patterns of restriction

fragments created by Hae III and Hpa II digestion. These results suggest that there exists in the mitochondrial DNA of various grande strains a certain amount of variability. It has been suggested (Sanders *et al.*, 1976) that some of this variability may be due to insertions or deletions of various sequences.

The Petite Mutant

In 1953, Ephrussi reported that when a population of yeast cells were plated onto a fermentable media, colonies of two distinct sizes were observed. The smaller colonies, later termed "petites", were found to be unable to utilize non-fermentable substrates, and to lack detectable amounts of cytochrome a, a_3 or b (Slonimski, 1953). The first paper describing the origin of the petite mutation reported that the induction of this mutation by acriflavine was unaffected by the ploidy of the yeast strains employed in this study (Ephrussi *et al.*, 1949). The independence of any nuclear gene dosage effect strongly suggested that this mutation is not occurring to the nuclear genome. Subsequent analysis of petite mutants has demonstrated that most transmit their defective genotype via the cytoplasm, presumably, the mitochondria (Wright and Lederberg, 1957), while few carry their mutation on the nuclear genome (Chen *et al.*, 1950).

The existence of two classes of cytoplasmic petites, termed neutral and suppressive were reported by Ephrussi and coworkers in 1955. The neutral petite is one which when crossed to a wild type grande tester strain produces diploids which are respiratory

competent. The suppressive petite, on the other hand, produces some respiratory incompetent diploid clones when mated to a wild type grande tester strain. The cause of the different phenotypic expressions by these two types of cytoplasmic petites is still not clear, however, recent reports have demonstrated that while the suppressive petites contain mitochondrial DNA many of the neutral petites lack any detectable mitochondrial DNA (Nagley and Linnane, 1970). In a recent report (Blamire _et al._, 1976) in which the fate of mitochondrial DNA, following a mating involving a suppressive petite and a grande strain was analyzed, it was found that the bouyant density of the mitochondrial DNA was seen to change in a manner which appeared to be the result of a recombination between these two mitochondrial DNAs followed by a modification of this DNA. These authors suggest that the phenomenon of suppressivity may be the result of the recombination of the altered mitochondrial DNA with the wild type mitochondrial DNA resulting in a recombined molecule containing altered genetic material.

Analysis of the mitochondrial DNA of cytoplasmic petites has demonstrated that this DNA often exhibits remarkable changes in its physical properties. The following changes have been observed: (1) base sequence alterations (Mounolou _et al._, 1966) in which in extreme cases the base composition may be changed from the wild type 17 mole % GC to 4-6% GC (so called low density petites) (Hollenberg _et al._, 1972) (2) loss of genetic complexity as measured

by renaturation kinetics (Michel *et al.*, 1974) (3) loss of mitochondrial genes that can be rescued in genetic crosses (i.e. antibiotic resistance markers) (Nagley and Linnane, 1972) (4) loss or repetition of genes that can be hybridized to mitochondrial ribosomal RNA (Fukuhara *et al.*, 1974) and transfer RNA (Cohen and Rabinowitz, 1972) (5) reiteration of petite mitochondrial DNA sequences often occurring in either tandem or inverted tandem arrangement (Locker *et al.*, 1974a) (6) presence of circular mitochondrial DNA molecules whose monomeric contour length can be correlated to the kinetic complexity of the petite mitochondrial DNA (Locker *et al.*, 1974b) and (7) altered electrophoretic patterns of the restriction fragments created by endonuclease digestion (van Kreijl and Bos, 1977).

Early investigators working with petite mutants suggested that all cytoplasmic petites possessed a basic mutational change of their mitochondrial DNA (or ρ factor as it was then referred to) which made complementation of petite strains to yield a grande diploid impossible (Roodyn and Wilkie, 1966). Recent work , however, has demonstrated that under conditions in which "early" cytoplasmic petite strains were mated,complementation yielding respiratory competent diploid cells resulted (Clark Walker and Miklos, 1975). This result suggests that the petite phenotype of the cytoplasmic mutant need not be the result of an alteration of a specific portion of the mitochondrial genome. These observations were further substantiated

by the work of Fukuhara and coworkers in which they demonstated, by means of hybridization analysis, that the respiratory incompetent phenotype of a cytoplasmic petite can be the result of a mutation of any portion of the mitochondrial genome (Bolotin-Fukuhara and Fukuhara, 1976).

Induction of Cytoplasmic Petites

The first paper on the origin of the petite mutation demonstrated that an entire population of yeast cells could be transformed from grande to petite (a process that occurs spontaneously at a frequency characteristic of each yeast strain) in the presence of the acridine dye, acriflavine (Ephrussi *et al*, 1949). Subsequently, it was demonstrated that it is the euflavine component of acriflavine that is responsible for the induction of the cytoplasmic petites (Marcovich, 1951). In 1954, Raut demonstrated that cytoplasmic petites could be induced by ultraviolet light irradiation. Among the other mutagenic agents found to have the ability to induce the cytoplasmic petite mutation are 5 flurorouracil (Moustacchi and Marcovich, 1963), 2, 3, 5 triphenyltetrazoluin (Laskowski, 1954), manganese, copper, cobalt and nickel (Lindegren *et al*., 1958) and ethidium bromide (Slonimski *et al*., 1968). Recent analysis has demonstrated that the cytoplasmic mutation can also be induced by hydrostatic pressure (Rosin and Zimmerman, 1977), fatty acid depletion of an unsaturated fatty acid requiring strain (Marzuki *et al*., 1974) and by the growth of strains harboring the cdc 8 and 21 lesions at the permissive temperature (Newlon and Fangman, 1975).

In 1956, Ycas demonstrated that growth of yeast at an elevated temperature induced petite mutations. Ycas found that at 40°C one could get the conversion of 100% of a yeast population to petite. In 1959, Sherman reported that when yeast cells grown at 30°C were shifted for a short time to 54°C petite induction occurred. In the past few years conditional mutants of yeast have been isolated which produce cytoplasmic petites at the non-permissive temperature at high frequencies (Weislogel and Butow, 1970; Handwerker *et al.*, 1973; Schweizer *et al.*, 1977 and Rubin and Blamire, 1977). One of these communications reports a turnoff of mitochondrial DNA synthesis at the non-permissive temperature (Rubin and Blamire, 1977).

Nuclear and Mitochondrial Interactions in the Assembly of the Mitochondria

The biogenesis of the mitochondria appears to be the result of a joint venture of both the nuclear and mitochondrial genetic systems. The contribution of the nuclear genetic system in the formation and maintenance of the mitochondria has been extensively studied with the use of cytoplasmic petite mutants of *Saccharomyces cerevisiae*. While different cytoplasmic petite mutants may retain varying amounts of mitochondrial DNA, all seem to have lost a functional mitochondrial protein synthesizing system and thus all proteins found in the mitochondria of a cytoplasmic petite must have been synthesized by the cytoplasmic ribosomes (Schatz and Mason, 1974). Analysis of petite mitochondria has demonstrated that they possess normal outer

membranes as well as a poorly developed inner membrane (Yotsuyanagi, 1962b). Among the proteins present in the petite mitochondria are all of the tricarboxylic acid cycle enzymes, parts of the respiratory chain, mitochondrial DNA polymerase as well as many other mitochondrial enzymes and structures (see Sager, 1972). The presence of a protein in mitochondria of a petite cell firmly establishes a nuclear coded origin of this protein. However, the absence of any protein need not mean that this protein is synthesized by the mitochondrial genetic system for the possibility exists that this protein is synthesized in the cytoplasm but requires a mitochondrial synthesized component for proper assembly into the mitochondria (Schatz and Mason, 1974). Because of this limitation in using cytoplasmic petites to identify the origin of specific mitochondrial proteins a second more flexible approach was undertaken by many investigators. This approach involves growing cells in the presence of inhibitors of mitochondrial and cytoplasmic protein synthesis and then determining which proteins are absent. In most of these analyses, the inhibitor of cytoplasmic protein synthesis employed was cycloheximide (So and Davie, 1968) while the inhibitors commonly used to block mitochondrial protein synthesis were erythromycin, chloramphenicol and lincomycin (Lamb *et al.*, 1968). By the appropriate use of inhibitors it has been estimated that between 85 to 95% of the total mitochondrial protein is made in the cytoplasm while the mitochondrial translation products have been estimated to account for only 5-15%

of the mitochondrial protein mass (Schweyen and Kaudewitz, 1970). Present evidence suggests that approximately ten hydrophobic polypeptides are synthesized by the mitochondria. These include three polypeptides of cytochrome oxidase (Mason and Schatz, 1977), four of the oligomycin sensitive ATPase (Tzagoloff and Meagher, 1972) and three of the cytochrome b complex (Marjanen and Ryrie, 1976).

The observation in 1969 that mitochondrial protein synthesis stops 30 to 60 minutes after exposure of the cells to cycloheximide (Sebald _et al._, 1969) was one of the earliest indications of the interdependence between cytoplasmic and mitochondrial protein synthesis in the formation of the mitochondria. This observation has been recomfirmed by many experiments performed to date (see Schatz and Mason, 1974). The coordination of the mitochondrial and cytoplasmic protein synthesis in mitochondrial assembly has been the subject of numerous studies. Kim and Beattie, in 1973, noted that the increase in activity of NADH-cytochrome c reductase and cytochrome oxidase upon derepression (a process that involves metabolic changes that occur to the mitochondria when cells growing anaerobically are exposed to oxygen), was completely inhibited by chloramphenicol but was not affected for several hours after the addition of cycloheximide. These results suggested that the synthesis of mitochondrial proteins on cytoplasmic ribosomes may precede the synthesis on the mitochondrial ribosomes and that these proteins may

accumulate in the mitochondria. This suggestion was confirmed by Mason and Schatz in 1973 in an experiment in which yeast cells labeled with ^{3}H-leucine in the presence of cycloheximide were lysed and found to contain cytochrome oxidase with radioactively labeled mitochondrial subunits. Since the cytoplasmic subunits could not have been synthesized after the addition of cycloheximide, they must have been present in the mitochondria in excess. Experiments with Neurospora crassa have also demonstrated an excess pool of some cytoplasmically made cytochrome oxidase subunits (Schwab et al., 1972). Several groups have reported that there occurs a stimulation of mitochondrial protein synthesis following a block of this synthesis with chloramphenicol (Tzagoloff, 1971; Ibrahim et al., 1973). This increased synthesis is believed to be the result of an accumulation of cytoplasmically synthesized proteins which in some way stimulates mitochondrial protein synthesis. Ibrahim and Beattie, in 1976, reported a possible mechanism for this regulation of mitochondrial protein synthesis by the cytoplasm. These investigators found that when yeast cells grown in chloramphenicol (to allow cytoplasmically made proteins to accumulate) were washed free of the chloramphenicol and allowed to grow for one hour in fresh media, a twofold increase in the mitochondrial polysome to monosome ratio was observed. Furthermore, these cells showed an increased incorporation of radioactive leucine into the polysome region of the mitochondria. In contrast, mitochondria isolated from cells which had

been pregrown in cycloheximide for three hours showed a 50% decrease in the polysome monosome ratio as well as in the amount of radioactive leucine incorporated into the polysome region. These results suggest the possibility that proteins synthesized in the cytoplasm may control mitochondrial protein synthesis either by stimulating chain initiation or by causing increased formation or stability of specific mitochondrial messenger RNAs (Ibrahim and Beattie, 1976).

The discussion thus far has dealt with the contribution of the nuclear genome in the formation of the mitochondria. Evidence presented in the last few years suggests that the mitochondria may play a role in the regulation of proteins synthesized under the control of nuclear genome. Treatment of *Tetrahymena* with ethidium bromide has been reported to induce the biosynthesis of mitochondrial DNA polymerase by the cytoplasm (Westergaard and Lindberg, 1972). In *Neurospora*, both chloramphenicol and ethidium bromide have been found to stimulate the production by the cytoplasm of mitochondrial elongation factors, methionyl-tRNA transfomylase and RNA polymerase (Barath and Kuntzel, 1972).

In a study by Puglisi and Algeri in 1974 using *Saccharomyces cerevisiae*, they examined the possibility that mitochondrial protein synthesis may play a role in nuclear gene activities. In their study they analyzed several cytoplasmic respiratory deficient mutants for their ability to induce enzyme production in response to galactose, an ability present in the parent strain of these mutants. These investigators found that approximately 50% of these cytoplasmic

mutants grew on galactose with only a delayed adaptation, while the remainder were unable to grow on this media at all. Since it is known that different petites can differ in terms of their mitochondrial DNA alterations, the experimenters treated the petite mutants with erythromycin and observed a complete cessation of the growth of all of the petite mutants on the galactose media. These results led the authors to propose a model in which a component of the regulatory system involved in galactose utilization is coded for by the mitochondria and thus suggested an active role of mitochondrial protein synthesis in the utilization of galactose. In order to determine the effect of the petite mutation on the fermentation of other carbon sources, Khan and Greener (1977) analyzed the effect of the petite mutation on maltose and alpha-methylglucoside fermentation in *Saccharomyces cerevisiae.* These investigators report that while most of the petite mutants isolated in this study have retained the ability to ferment maltose and alpha-methylglucoside, the petites isolated from one strain have completely lost their ability to ferment alpha-methylglucoside, thus suggesting an involvement of a mitochondrial factor in the utilization of alpha-methylglucoside in this strain.

In 1951, during a study on the process of sporulation, Ephrussi and Hottinguer noted the inability of petite diploids to undergo sporulation and thus suggested a possible involvement of the mitochondrial genome in this developmental process. In 1956, Miller and Halpern reported the failure of diploid cells to undergo sporulation in the

presence of respiratory inhibitors. Puglisi and Zennaro, in 1971, studied the effect of erythromycin on the sporulation of erythromycin sensitive and erythromycin resistant a/α diploids and found that erythromycin inhibits sporulation of erythromycin sensitive diploids but does not block sporulation in its erythromycin resistant derivative. These results suggested that the inhibition of sporulation by erythromycin reflects the dependence of this process on mitochondrial protein synthesis. In a recent communication by Kuenzi and coworkers (1974), this suggestion has been questioned. These investigators report that petite diploid cells can, in fact, be made to sporulate if they are transferred to sporulation media shortly after being mutated to the petite state. These results suggest the possibility that it is not mitochondrial protein synthesis which is required for sporulation but the full respiratory activity of the cells.

The second study reported in this communication, in which a temperature sensitive mutant of *Saccharomyces cerevisiae* defective in nuclear DNA metabolism is described, was undertaken to determine if there exist genetic loci other than the cdc loci (see Hartwell, 1974) which are required for accurate nuclear DNA metabolism. Our selection for such mutants involved the isolation of strains which at the non-permissive temperature induced lethality. The isolation and characterization of a mutant defective in nuclear DNA metabolism is described.

Temperature Sensitive Mutants of Nuclear DNA Metabolism in *Saccharomyces cerevisiae*

In 1951, Horowitz and Leupold , at a symposium at Cold Spring Harbor Laboratory, suggested that a method for detecting mutations which result in the loss of an indispensible function might be obtainable through so called "temperature mutants" in which the expression of a gene is normal at one temperature but abnormal at another. Edgar and coworkers (1963, 1964), working with the bacteriophage T_4, demonstrated that such temperature sensitive mutations can in fact occur in the nondispensible functions and can thus be used to gain valuable information about these genes. Hartwell, in 1967, following a report by Neidhardt (1964) on the isolation, characterization, and patterns of macromolecular synthesis of temperature sensitive mutants of *Escherichia coli*, decided to analyze a eukaryotic system to see if such temperature sensitive lesions can be induced in eukaryotes. For his study, Hartwell selected *Saccharomyces cerevisiae*, for it is one of the simplest eukaryotic organisms having only 2.2×10^{-8}μg of DNA per haploid nucleus (Ogur *et al.*, 1952) and having a genetic system allowing for an analysis of the meiotic products, a study of complementation patterns, and a determination of dominance and recessiveness of various mutations. After mutagenizing a culture of strain A364A with N-methyl-N'-nitoso-N-nitrosoguanidine, the culture was plated out and incubated at 23°C. When colonies appeared on the plates, they were replicaplated onto two new plates which were incubated at 23°C or 36°C. Colonies which grew on the former but not

on the latter were further screened for their temperature sensitivity. Approximately 400 temperature sensitive mutants were isolated in this fashion. This set of 400 mutants ultimately yielded 5 genes (cdc 4,7,8, 21,28) with lesions in genes apparently involved in DNA replication or its initiation(Hartwell, 1973). Mutations affecting protein synthesis and RNA synthesis were also found. The 400 mutants in Hartwell's collection were also examined microscopically to determine cell morphology after incubation at the restrictive temperature. This analysis revealed the existence of 14 genes (designated cly 1-14) in which the cells lyse shortly after the transfer to the restictive temperature as well as the existence of mutants defective in specific steps in the cell cycle. At the present time, 35 such genes (cdc 1-35) have been identified.

The patterns of DNA synthesis of the cell division cycle mutants defective in DNA synthesis were examined in synchronous and asynchronous cultures to try and classify the point at which these genes have their execution points. The kinetics of DNA synthesis after the shift to the restrictive temperature was compared to that obtained after inhibition of protein synthesis at the permissive temperature, a condition that specifically blocks the inhibition of new rounds of DNA replication. but does not block those rounds of replication that are in progress (Hereford and Hartwell, 1973).

Three of the cdc mutants (cdc 4,7, and 28) appear to result in a block at the start of new rounds of DNA synthesis, after the shift to the restrictive temperature, while allowing the completion of those rounds

of replication that are in progress. These gene products were thus classified as being essential for the initiation of DNA synthesis. Mutations of two of the cdc genes (cdc 8 and 21) appear to block the actual synthesis of the DNA, for it has been demonstrated that there is an abrupt halt of DNA synthesis after the shift to the restrictive temperature. These gene products were classified as being essential for the synthesis of the DNA (see Hartwell, 1973).

DNA molecules in the process of replication contain "bubbles" and "forks" that can be visualized by electron microscopy (Newlon *et al.*, 1974). Petes and Newlon, in 1974, studied the structure of the DNA of cells carrying the cdc 7 and the cdc 8 lesions after the shift to the restrictive temperature. They found that while the DNA of cells containing the cdc 8 lesion contained multiple small "bubbles", the DNA of the cells containing the cdc 7 lesion contained very few "bubbles". These results are interpreted to mean that the cells with the cdc 7 mutation are in fact arrested prior to the actual beginning of chromosome replication, while cells containing the cdc 8 mutation are blocked during replication. Because the cdc 4 and cdc 8 mutation have been found to precede the cdc 7 mediated step of DNA synthesis, it appears to confirm the suggestion that these gene products are required for DNA initiation (Hereford and Hartwell, 1974).

MATERIALS AND METHODS

Yeast Strains

The strains of Saccharomyces cerevisiae used in this paper constitute part of our laboratory stock strains and are listed with their genotypes in Table 1.

Media

YEP media: This was used as the basis of most media in this work. It consists of 1% Difco Yeast Extract, 2% Difco Bacto Peptone, and 2% Difco Bacto Agar when solid media was required.

YEPD media: consists of YEP media plus 2% Bacto Dextrose.

YEPG media: consists of YEP media plus 3% Bacto Glycerol.

YEPGal media: consists of YEP media plus 2% Bacto Galactose.

YEP-Dif media: consists of YEP media plus 3% Bacto Glycerol and 0.1% Bacto Dextrose. This media was used to differentiate colonies arising from grande cells from those arising from petite cells on the basis of their colony size.

YNB media: This was used as the basis of all minimal media. It contains 0.67% Difco Bacto Yeast Nitrogen Base without amino acids and 2% Difco Bacto Agar when solid media was required.

YNBD media: consists of YNB media plus 2% Bacto Dextrose.

YNBG media: consists of YNB media plus 3% Bacto Glycerol.

YNBGal media: consists of YNB media plus 2% Bacto Galactose.

YNBD/G/Gal + X media: This was used for radioactive labeling in which the YNB was supplemented with the auxotrophic requirements

of the various strains. The supplementation was accomplished with the addition of any of the following: adenine, 30mg/l; L-arginine, 30mg/l; L-histidine, 20mg/l; L- leucine, 40 mg/l; L-lysine, 40 mg/l; L-methionine, 20 mg/l; DL-threonine, 150 mg/l; L-tryptophan, 30 mg/l; L-tyrosine, 20 mg/l; uracil, 20 mg/l.

CS media: Consists of YNBD media supplemented with all of the above mentioned supplements.

Drop out media: Consists of CS media lacking one or more supplements.

YNBDif + X: consists of supplemented YNB media plus 3 % Bacto Glycerol and 0.1% Bacto Dextrose.

KAc media: Consists of 0.96% Potassium acetate. 0.1% Bacto Dextrose, 0.25% Difco Yeast Extract and 2% Difco Bacto Agar. This media was used to induce sporulation (MacKay and Manney, 1974).

Dissecting Agar: Consists of 0.25% Difco Yeast Extract, 0.05% Bacto Dextrose and 2% Difco Bacto Agar.

Mutagenesis

Cultures of strain 123.1C grown at 24°C in liquid YEPD media to stationary phase were harvested by centrifugation and resuspended into 1.9 ml of 0.1M phosphate buffer (pH 6.8) to which 50-100 ug/ml of ethyl-methane sulphonate (EMS) was added. After 80-90 min. of exposure to the EMS,the cells were harvested, resuspended in 8 ml of 5% sodium thiosulfate, vortexed, and allowed to incubate for 10 min. at 24°C. Following this incubation period the cells were harvested, washed in 5 ml of YEPD media, reharvested and resuspended in 5 ml of fresh

YEPD media. Potential mutants were allowed to grow for two hours at 24°C and were then diluted and transferred onto solid YEPD plates. Surviving clones were screened as described in the results.

Tetrad Analysis

Diploids isolated on selective media were transferred to KAc sporulation media and incubated at 24°C for five days. The ascus walls of the resulting meiotic products were partially digested away by the incubation of the asci for ten to fifteen minutes at 24°C in the presence of 1% glusalase (Endo Laboratories). The asci were then transferred to a slab of dissecting agar and the individual spores micromanipulated to defined positions on the slab. The slab of dissecting agar was then placed onto a YEPD plate and allowed to incubate for seven to nine days at 24°C. The resulting clones were then transferred onto "master plates" of YEPD media and allowed to grow for five days at 24°C (see Mortimer and Hawthorne 1969).

The auxotrophic requirements of each spore was determined by replica-plating the "master plates" onto various dropout media and scoring for the ability or inability of each clone to grow after five days of incubation at 24°C.

The mating type of each spore was determined as follows: On day one, separate YEPD plates were plated with approximately 10^6 cells of either a or α mating type tester strains and allowed to incubate at 30°C for twenty-four hours. On day two, the mating tester "lawns" were replica-plated onto the surface of separated YEPD plates together with a replica of the master plate and allowed to incubate at 24°C for twenty-four hours.

On day three, these plates were rereplicaplated onto YNBD or YNBD + X media so that only the resulting diploids would be able to grow. Confluent growth on the selective media at any intersection after four days of incubation at 24°C was taken as a positive result and the spores assigned their appropriate mating types. Spores not mating to either "a" or "α" mating type tester strains or mating to both strains at very low frequencies were classified as being sterile.

Monitoring of Cell Growth

Cell growth was monitored by optical density measurements and viable count determinations. Optical density measurements were obtained by means of a Klett-Summerson colorimeter fitted with a 660 nm filter. Viable count determinations were accomplished by diluting and transferring cells onto solid YEPD media and counting the number of colonies formed on this media after approximately six days of growth at 24°C.

Determination of Suppressivity in Petite Strains

Petites grown to late log phase were mixed with a grande tester strain in liquid YEPD and plated out on YNBDif + X prior to the release of the first bud from the diploid zygotes. After approximately nine days the percentage of petite diploid colonies was determined on the basis of the size of the colonies. The formula $\frac{X-Y}{100-Y}$ was used to calculate the suppressivity, where X equals the percentage of petite diploids and Y the percentage of petites in the tester strain (Jakob, 1962).

Radioactive Labeling of Cultures

The labeling of nucleic acids was accomplished with either 2-^{3}H-adenine or ^{14}C-adenine (New England Nuclear). Concentration of label used ranged from 15-20 µCi ^{3}H-adenine per ml of culture and 0.5 - 1.5 µCi of ^{14}C-adenine per ml of culture. Cultures were grown in YNB D/G + X and labeled in mid to late log phase.

In experiments in which preferential labeling of mitochondrial DNA was desired, 200 µg/ml of cycloheximide was added to the culture concurrently with radioisotope in order to inhibit nuclear DNA synthesis and allow for increased incorporation of the isotope into the mitochondrial DNA (Grossman *et al.*, 1969).

Determination of DNA Synthesis

DNA synthesis was monitored via the incorporation of 2-^{3}H-adenine into hot alkali stable cold acid precipitable material. Triplicate 0.5 ml samples of a radioactively labeled culture, treated with 0.5 ml of a 0.5M KOH and 20% dimethylsulfoxide solution, (Bartolome and Orrego, 1970) were hydrolyzed for two hours at 60°C. These samples were then precipitated with 1 ml of 20% cold trichloroacetic acid (TCA), chilled to 4°C for 30 minutes and collected onto Whatman GF/A glass fiber filters. These filters were then washed twice with cold 5% TCA, once with 95% ethanol, dried and placed into vials to which 4 ml of toluene bases scintillant was added (Liquorflour, New England Nuclear). Samples were counted in a Nuclear Chicago Liquid Scintillation Spectrometer (Michels *et al.*, 1973).

Preparation of Spheroplasts

Preparation of spheroplasts from small quantities of cells (less than one gram) was accomplished as follows: cells harvested by centrifugation at 5000 x g for 5 minutes were resuspended in 5 ml of thioglycollate buffer (0.5 M sodium thioglycollate in 0.1 M Tris, pH 8.8) and allowed to incubate for an hour at 30°C with continual shaking. Following this incubation period the cells were harvested, washed in 2 ml of sorbitol buffer (1 M sorbitol, 0.1 M disodium ethylenediaminetetraacetate), reharvested, and resuspended in 1 ml of sorbitol buffer. Digestion of the cell walls was then accomplished by the incubation of the cells for 30 minutes at 30°C in the presence of 1-2% Glusulase (Endo Laboratories). The spheroplasts were then gently harvested at 1000 x g for 5 minutes, resuspended in 1.4 ml saline EDTA buffer (0.15 M NaCl, 0.1 M disodium ethylenediaminetetraacetate, pH 8.0), and lysed at 60°C with approximately 3% Sarkosyl NL-97 (Ciba-Geigy, Co.). Samples were allowed to incubate at 60°C for 20 minutes after which they were stored at -20°C for subsequent use (Blamire et al., 1972a).

Preparative CsCl Density Gradient Centrifugation

Lysates (1.5 ml) were transferred to cellulose nitrate ultracentrifuge tubes (Beckman) containing 4.1 ml of stock preparative grade CsCl (Kawecki Beryl Co. Industries, Inc.) solution (130 g of CsCl in 70 ml of 0.01 M Tris buffer, pH 6.8), and approximately 1000 cpm of ^{14}C marker DNA from strain 123.1C was added. The density was adjusted

to 1.695 g/cm^3 with the use of a refractometer and mineral oil was added to fill the tube. Samples were centrifuged at 33,00 rpm in a Spinco 50 Ti rotor for at least 42 hours at 19°C. Fractions of 0.2 ml were collected from the bottom of the gradient (approximately 25 fractions per gradient). Each fraction was adjusted to 0.5 M KOH and hydrolysis of the RNA was allowed to take place either at 60°C for 2 hours or at 30°C for 18 hours. Following this incubation period 100 µg of Bovine serum albumin (BSA) was added to each fraction and the KOH was neutralized with 20% trichloroacetic acid (TCA). Excess TCA was added to a final concentration of 5% and the samples cooled to 4°C for 30 minutes. The precipitates were then collected on Whatman GF/A glass fiber filters, washed twice with cold 5% TCA, once with 95% ethanol, dried and placed into vials to which 4 ml of toluene based scintillant was added (Liquorfluor, New England Nuclear). Samples were counted in a Nuclear Chicago Liquid Scintillation Spectrometer (Michels _et al._, 1973).

Determination of the M/T Ratio as a Percent of the 24°C Control

The sum of the incorporation into alkali stable cold acid precipitable material banding in isopycnic CsCl density gradients at the position of the mitochondrial DNA was divided by the total number of counts incorporated into all species of DNA banding in the CsCl gradient (M/T). This ratio was then compared to the M/T ratio obtained from the 24°C control and calculated as a percent of the 24°C control M/T ratio by means of the formula M/T ratio of 36°C / M/T ratio of 24°C x 100.

RESULTS

Chapter 1. Isolation and Characterization of a Mutant Conditionally Deficient in Mitochondrial DNA Metabolism

Isolation, Phenotype and Genetic Analysis

A stationary culture of strain 123.1C was mutagenized for 90 minutes at 24°C with 50 µg/ml of ethylmethane sulphonate (EMS) in 0.1 M phosphate buffer (pH6.8). Potential mutants were diluted, transferred onto solid YEPD plates and incubated at 24°C for approximately 6 days. The colonies formed were then replicaplated onto 4 more plates as follows: 1) YEPD plated which was then incubated at 24°C; 2) 1 YEPD plate which was then incubated at 36°C; 3) 1 YEPG plate which was then incubated at 24°C and; 4) 1 YEPG plate which was then incubated at 36°C. Ninety six clones unable to grow on plate #4 but able to grow on the three remaining plates were taken and tested for petite induction at the restrictive temperature of 36°C. This was accomplished by growing the cells at 36°C in 5 ml of liquid YEPD media for 24 hours and then assaying the growth of the population of cells on YEP-Dif media at 24°C. Mutant strain TS-1G-95b was found to induce petites at a high frequency under these conditions and was chosen for further analysis.

Diploids from a cross between strain TS-1G-95b and CR-9B, isolated on selective media, were induced to undergo meiosis on KAc media at 24°C (see Materials and Methods). Tetrad analysis performed on these diploids produced spores which showed the same temperature sensitive petite inducing phenotype as strain TS-1G-95b. One of these mutant spores (144-2C) was then chosen for all subsequent

work. Strain 144-2C was then mated to strain 103.1A and the diploids which were isolated on selective media as before were made to under go meiosis. Analysis of the 94 asci dissected showed a clear 2:2 segregation for the mutant gene as well as for all of the other genetic markers, thus demonstrating that the mutant phenotype is the result of a single nuclear mutation.

Two diploid strains, x463 and x464, were created by mating the temperature sensitive strain 144-2C to the non-temperature sensitive laboratory stock strains 115.8C and 103.1D respectively. These diploids, isolated on selective media, were grown in liquid YEPD media to mid log phase and then diluted into YEPD media at a concentration of 1.57×10^3 and 1.4×10^3 cells per ml respectively. Equal aliquots of the diluted cultures were then incubated at 24°C or 36°C for approximately fifteen generations. During this period, petite induction was monitored at both the permissive and restrictive temperatures by diluting and transferring the cells onto solid YEP-Dif media. As can be seen in Figure 1, these diploid strains heterozygous for the petite inducing mutation, fail to induce a significant number of petites at the restrictive temperature, thus demonstrating that the mutation harbored in strain 144-2C is of a recessive nature.

Growth of Strain 144-2C in Media Containing Dextrose as its Sole Carbon Source

A culture of cells of strain 144-2C growing in liquid YEPD at 24°C was diluted in YEPD mediato a concentration of 5×10^3 cells per ml. Equal aliquots of the diluted culture were incubated at either 24°C or

$36^{o}C$ and the cells were allowed to grow and divide for six generations. Petite induction was monitored throughout this period at both the permissive and restrictive temperatures by diluting and transferring the cells onto solid YEP-Dif media. As can be seen (Figure 2), cells grown at $36^{o}C$ produced over 95% petites in six generations while induction of petites at $24^{o}C$, though present, occurred at a much lower frequency. Petite clones isolated from strain 144-2C after exposure to $36^{o}C$ for varying lengths of time were then tested for the following two properties: 1) the suppressive nature of these cells, and 2) the ability of the petite clones to synthesize mitochondrial DNA at the permissive temperature. Suppressivity was determined as described in the Materials and Methods. A cross between the petite clones and a grande tester strain(115.8C) resulted in 0% suppressiveness. Thus the petites induced at $36^{o}C$ are of a neutral nature. In testing for the ability of the petite clones to synthesize mitochondrial DNA, several of the petite clones induced at $36^{o}C$ were allowed to grow for 4 hours at $24^{o}C$ in YNBD + X media to which 200 μg/ml of cycloheximide and 20 μCi/ml of ^{3}H-adenine were added (see Materials and Methods). These cells were then harvested, lysed, and their DNA synthesis profiles monitored by preparative CsCl density gradient centrifugation. All 10 clones tested in this fashion gave radioactive DNA banding profiles as shown in Figure 3. It can be seen that there has been no incorporation of the isotope into the mitochondrial DNA even in the presence of cycloheximide. The DNA banding profile

presented suggests that there is no mitochondrial DNA in the petites induced at 36°C, thus confirming the neutral nature of these petites.

Synthesis of Mitochondrial DNA in Strain 144-2C at 24°C and 36°C in Media Containing Dextrose as its Sole Carbon Source

In order to determine the extent of mitochondrial DNA synthesis of strain 144-2C at the permissive and restrictive temperatures, the following experiment was performed. A culture of 144-2C grown for 15 hours in liquid YNBD + X at 24°C was divided into two equal aliquots. These cultures were then incubated at 24°C or 36°C in the presence of 20 µCi of ^{3}H-adenine per ml. After the 4 hour incubation period, lysates were prepared as before and the radioactive DNA banding profiles of these cultures monitored by preparative CsCl density gradient centrifugation (Figures 4a and 4b). A comparison between these two profiles reveals that the relative incorporation of radioactive adenine into the mitochondrial DNA species is reduced at the non-permissive temperature. In order to further resolve the differences in DNA synthesis patterns between cells grown at 24°C or 36°C, a similar experiment to the one described above was performed. Two equal aliqots of strain 144-2C, treated concurrently with 200 µg/ml of cycloheximide and 20 µCi/ml of ^{3}H-adenine, were incubated either at 24°C or 36°C. An analysis of the DNA banding profiles of these two cultures (Figures 4c and 4d) supports the notion that the relative incorporation of radioactive adenine into the mitochondrial DNA of strain 144-2C is significantly reduced at 36°C. As

a control to the above described experiments the DNA banding patterns of the parental strain (123.1C) of the original mutant was monitored at 24°C and 36°C in the absence of cycloheximide (Figures 4e and 4f). Analysis of this pair of profiles demonstrates that mitochondrial DNA synthesis comprises a larger proportion of the total DNA synthesized at 36°C than at 24°C. A related phenomenon has previously been reported for strain A364A (Hartwell, 1973), when it was demonstrated that the rate of DNA synthesis in this strain, as measured by TCA precipitable alkali resistant counts, decreased transiently after the transfer of the culture to 36°C. The data presented here (Figures 4e and 4f) suggest that the transient inhibition may result from a reduction in the synthesis of the nuclear DNA.

A culture of strain 144-2C grown in the presence of 20 μCi/ml of ^{3}H-adenine for 15 hours at 24°C was harvested, resuspended in fresh non-radioactive YNBD + X media and divided into two equal aliquots. These were then incubated for 4 hours at either 24°C or 36°C. The DNA of these two cultures was analyzed by preparative CsCl density gradient centrifugation. Figures 5a and 5b represent the results of this experiment in which it can be seen that there is little or no degradation of prelabeled mitochondrial DNA at 36°C. This result suggests that the nature of the temperature sensitive petite inducing phenotype observed in this strain in media containing dextrose as its sole carbon source, is due to the reduction or absence of mitochondrial DNA synthesis at the restrictive temperature

and not due to a degradation of preexisting mitochondrial DNA.

Growth of Strain 144-2C in Media Containing Glycerol as its Sole Carbon Source

A culture of 144-2C grown at 24°C in liquid YEPG was diluted and transferred into two tubes of fresh media at a concentration of 2.5×10^4 cells per ml. These cultures were then incubated at 24°C or 36°C and petite induction monitored by diluting and transferring the cells onto solid YEP-Dif media for seven generations (Figure 6). As can be seen in Figure 5, growth of strain 144-2C in liquid YEPG media at the restrictive temperature produced over 50% petites in seven generations while 97% of the cells grown at 24°C remained respiratory competent. Petite clones isolated after varying lengths of exposure to 36°C were tested for their suppressivity and for their ability to synthesize mitochondrial DNA at the permissive temperature. The petite clones mated to strain 115.8C and transferred onto solid YNB-Dif + X were found to be 2-52% suppressive (see Materials and Methods). The ability of these petite clones to synthesize mitochondrial DNA at the permissive temperature was examined as follows: 15 petite clones, grown at 24°C in liquid YNBD + X media for 15 hours, were treated simultaneously with 200 µg/ml cycloheximide and 20 µCi/ml of ^{3}H-adenine and allowed to incubate for 4 hours. The cells were then harvested, lysed, and their DNA synthesis profile monitored by preparative CsCl density gradient centrifugation. Figure 7 represents a typical banding profile of

the petite clones displaying synthesis of mitochondrial DNA. The existence of mitochondiral DNA in these petites corroborates the suppressive nature of these cells.

Synthesis of Mitochondrial DNA in Strain 144-2C at 24°C and 36°C in Media Containing Glycerol as its Sole Carbon Source

In order to determine if there was any reduction in the synthesis of mitochondrial DNA at the restrictive temperature in media containing glycerol as its sole carbon source, a culture of strain 144-2C grown in YNBG + X for 14 hours at 24°C was divided into two equal aliquots and labeled with ^{3}H-adenine for 4 hours at either 24°C or 36°C. These cells were then harvested, lysed, and their DNA synthesis patterns examined as before. Figures 8a and 8b represent the banding profiles of these DNAs in which it can be seen that the restrictive temperature appears to have no affect on the relative incorporation of ^{3}H-adenine into the mitochondrial DNA species. When the DNA synthesis pattern of the parental strain (123.1C) of the original mutant was examined at the permissive and restrictive temperatures in the same manner as above, it was found (Figures 8c and 8d) that the relative incorporation of radioactive adenine into the mitochondrial genomes was identical. These results suggest that the synthesis of mitochondrial DNA during this 4 hour labeling period by strain 144-2C at the restrictive temperature in media containing glycerol as its sole carbon source occurs at near normal levels.

Phenotypic Expression of the 144-2C Mutant Gene Product in Media Containing Galactose as its Sole Carbon Source

The different phenotypic expressions of the mutant strain 144-2C when grown on dextrose containing media versus glycerol containing media was thought to, perhaps, be due to the repressing and derepressing natures of these carbon sources on the mitochondria. Thus, experiments were performed to determine the phenotypic expression of this temperature sensitive mutant in media containing galactose, a fermentable and probably non-repressing carbon source (Tustanoff and Bartley, 1964).

1. Growth of Strain 144-2C in Media Containing Galactose as its Sole Carbon Source

An isolate of strain 144-2C which had been selected for its ability to utilize galactose as a carbon source (144-2C Gal^{+}) was grown at 24°C to mid log phase in liquid YEP-Gal. This culture was then diluted into fresh YEP-Gal media at a concentration of 3.2×10^{4} cells per ml. Equal aliquots of the diluted cultures were then incubated at either 24°C or 36°C and the cells allowed to grow and divide for seven generations. Petite induction was monitored throughout this period at the permissive and restrictive temperatures by diluting and transferring aliquots of the experimental cultures onto solid YEP-Dif media. As can be seen in Figure 9, cells grown at 36°C produced 90% petites in seven generations while induction of petites at 24°C occurred at a much lower frequency. Petite clones isolated from strain 144-2C Gal^{+}

after exposure to 36°C for varying lengths of time were then tested for the following two properties: 1) the suppressive nature of the clones, and 2) the ability of these clones to synthesize mitochondrial DNA at the permissive temperature. Suppressivity was determined as described previously (see also Rubin and Blamire, 1977). A cross between several of the petite clones isolated after 2.5 generations of exposure to 36°C and a grande tester strain (103.1A) resulted in 0% suppressiveness. This suggested that the petites induced at 36°C in galactose containing media would not contain mitochondrial DNA. In order to test this possibility, the capacity of the petite clones to synthesize mitochondrial DNA was measured as previously described. Nine petite clones induced following 2.5 generations of growth at 36°C were allowed to grow for four hours at 24°C in YNBD + X media to which 200 µg/ml of cycloheximide and 20 µCi/ml of ^{3}H-adenine were added. These cells were then harvested, lysed and their DNA banding profiles monitored by preparative CsCl density gradient centrifugation. All of the nine clones tested in this fashion gave radioactive DNA banding profiles similar to that shown in Figure 10. It can be seen that there is no incorporation of radioactive adenine into mitochondrial DNA even in the presence of cycloheximide. The above results suggest, as has been found to be the case with the petite cells induced at 36°C by strain 144-2C grown in dextrose containing media, that there is no mitochondrial DNA in the petites induced at 36°C in galactose containing media and that the petite cells induced are of a neutral nature.

2. Synthesis of Mitochondrial DNA in Strain 144-2C at 24°C and 36°C in Media Containing Galactose as its Sole Carbon Source

In order to determine the extent of mitochondrial DNA synthesis of strain 144-2C Gal$^+$ at the permissive and restrictive temperatures, the following experiment was performed. A culture of 144-2C Gal$^+$ grown in YNBGal + X for fourteen hours at 24°C was divided into two equal aliquots and treated with 20 µCi/ml ^{3}H-adenine for four hours at either 24°C or 36°C. After this four hour incubation period, lysates were prepared and the radioactive DNA banding profiles of these cultures monitored by preparative CsCl density gradient centrifugation (Figures 11a and 11b). A comparison between these two profiles reveals that the relative incorporation of radioactive adenine into the mitochondrial DNA is reduced at the non-permissive temperature. In order to further resolve the difference in the relative incorporation of ^{3}H-adenine into the mitochondrial DNA, an experiment similar to the one described above was performed. Two equal aliquots of strain 144-2C were treated concurrently with 200 µg/ml of cycloheximide and 20 µCi/ml of ^{3}H-adenine and then allowed to incubate at either 24° or 36°C for a four hour period. An analysis of these DNA banding profiles (Figures 11c and 11d) demonstrates that a significant reduction in the relative incorporation of radioactive adenine into the mitochondrial DNA at the elevated temperature has taken place. The above results suggest, as has been found to be the case when strain 144-2C is grown in dextrose containing media (Rubin and Blamire, 1977), that the relative incorporation of radio-

active adenine into the mitochondrial DNA is significantly reduced at the restrictive temperature. An isolate of the parental strain (123.1C), from which the temperature strain was derived, was selected for, which had the ability to utilize galactose as a carbon source (123.1C Gal^+) and then used as a control for the above experiments. The DNA banding profiles of this strain were monitored after a four hour labeling period at 24°C and 36°C in galactose containing media (Figures 11e and 11f). Analysis of this pair of profiles demonstrates that the relative incorporation at the two temperatures into the mitochondrial DNAs is nearly identical.

An Analysis of the Kinetics of Mitochondrial DNA Synthesis at the Elevated Temperature in Media Containing Either Dextrose or Glycerol as its Sole Carbon Source

In order to determine the rate at which mitochondrial DNA synthesis occurs after various lengths of exposure to the restrictive temperature, the following experiment was performed. Thirty ml cultures of strain 144-2C grown for twelve hours in liquid YNBD + X media or fourteen hours in YNBG + X media at 24°C were divided, respectively, into four 5 ml aliquots. Prior to the radioactive labeling of these cells the samples were incubated as follows: culture 1; incubated at 36°C for four hours, culture 2; incubated at 24°C for two hours and 36°C for the subsequent two hours, and cultures 3 and 4; allowed to continue incubating at 24°C for this entire four hour period. After these preincubation periods, cultures 1, 2, and 3 were treated with ^3H-adenine

for an additional two hour period at 36°C, while culture 4 was similarly labeled at 24°C for the same two hour period. These cells were then harvested, lysed and their DNA banding patterns examined as before. The amount of radioactive incorporation into the mitochondrial DNA is expressed as a percent of the mitochondrial/total ratio of the 24°C control (see Materials and Methods). As can be seen in Figure 12a, the relative incorporation into the mitochondrial DNA of cells grown on YNBD + X media is reduced to approximately 28% of the 24°C control after two hours of preincubation at 36°C. The increased incorporation into the mitochondrial DNA during the initial two hours following the transfer to the elevated temperature has been discussed previously (Rubin and Blamire, 1977). The relative incorporation into the mitochondrial DNA of cells grown on YNBG + X media was found to be reduced to approximately 30% of the 24°C control after two hours of preincubation at 36°C (Figure 12b). Analysis of the relative incorporation into the mitochondrial DNA of the parental strain of this mutant (123.1C) grown at 36°C in either YNBD + X or YNBG + X media was found to be approximately 100% of its 24°C control even after six hours preincubation at 36°C (data not shown). The reduction in the relative incorporation of radioactive adenine into the mitochondrial DNA of cells incubated in glycerol media for varying lengths of time at the restrictive temperature is different than that observed in the experiment in which radioactive incorporation for the initial four hours following the transfer to 36°C was found to occur

at a level comparable to that of the 24°C control (see Discussion).

Phenotypic Expression of the 144-2C Mutant Gene Product in a Respiratory Incompetent Cell

In order to determine if the expression of the 144-2C mutant gene product is dependent on the respiratory competence of the mutant cell, the following experiment was performed. A spontaneous petite, containing mitochondrial DNA and expressing 30% suppressivity, was isolated from strain 144-2C at the permissive temperature (144-2C ρ^--3). A culture of this petite strain grown at 24°C to mid log phase in liquid YEPD media was diluted into YEPD media at a concentration of 5.6×10^3 cells per ml. Equal aliquots of the diluted culture were incubated at 24°C or 36°C for approximately 32 hours. During this period, the growth of the cells was monitored by diluting and transferring aliquots of the experimental cultures onto solid YEPD, and the number of cells able to form colonies determined after seven days of incubation at 24°C (Figure 13). Clones isolated from strain 144-2C ρ^--3 after ten hours or more of exposure to 24°C or 36°C were tested for their suppressivity and for their ability to synthesize mitochondrial DNA at the permissive temperature. The six petite clones isolated after growth at 24°C were mated to strain 103.1A and found to range in suppressivity from 32% to 46%, while all of the ten clones isolated from the culture incubated at 36°C exhibited 0% suppressivity. The ability of these petite clones to synthesize mitochondrial DNA at the permissive temperature was examined as follows: six petite clones isolated after eight hours or more of growth at 24°C

and ten petite clones isolated after ten hours or more of growth at 36°C were grown for fifteen hours in liquid YNBD + X and then treated with 200 µg/ml cycloheximide and 10 µCi/ml of ^{3}H-adenine. These cultures were allowed to incubate for four hours after which the cells were harvested, lysed and their DNA banding profiles monitored by preparative CsCl density gradient centrifugation. All of the six clones isolated after growth at 24°C gave radioactive DNA profiles similar to that shown in Figure 14a in which it can be seen that there has been incorporation of isotope into both the nuclear and the mitochondrial DNA. However, analysis of the clones isolated after growth at 36°C gave radioactive DNA profiles in which there was no incorporation of radioactive isotope into the mitochondrial DNA even in the presence of cycloheximide (Figure 14b). These results suggest that the cells grown at 36°C no longer contain mitochondrial DNA while those cells grown at 24°C still contain mitochondrial DNA, thus establishing that the expression of the petite inducing phenotype is not dependent on the respiratory capacity of the mutant cell.

Chapter 2. Isolation and Characterization of a Mutant Conditionally Deficient in Nuclear DNA Metabolism

Isolation, Phenotype and Genetic Analysis

A stationary phase culture of strain 123.1C was mutagenized at 24°C with 75 µg/ml ethylmethane sulphonate (EMS) in 0.1M phosphate buffer (pH 6.8) for 80 minutes.

Potential mutants were diluted in sterile distilled water, transferred to solid YEPD plates and allowed to incubate at 24°C for 6 days. The resulting colonies were replicaplated onto two identical YEPD plates, one of which was then incubated at 24°C and the other at 36°C. Those colonies able to grow at 24°C but not at 36°C were then selected for further analysis. These potential mutants were analyzed by growing them in liquid YEPD media at 24°C to mid log phase and then transferring them to 36°C for a 24 hour period. Following this exposure to the elevated temperature, the number of viable cells was determined by diluting and plating the experimental culture onto solid YEPD media and monitoring the ability of the cells to form viable colonies at 24°C (see Materials and Methods). Mutant strain TS6M4.6 was found to exhibit a very rapid loss of viability following exposure to the elevated temperature and was thus chosen for further analysis.

A diploid strain, x 403, was produced by mating temperature sensitive strain TS6M4.6 to the non-temperature sensitive laboratory stock strain 102.10A. This diploid was induced to undergo meiosis by transferring to KAc media (see Materials and Methods). Thirty two of the resulting asci were analyzed by tetrad analysis and were found to ex-

hibit a clear 2:2 segregation for the mutant gene as well as for all of the other genetic markers, thus demonstrating that the mutant phenotype of this strain is the result of a single nuclear gene mutation.

The recessiveness of the temperature sensitive phenotype was determined by subjecting equal aliquots of a culture of strain x403, previously grown to mid log phase in liquid YEPD, to incubation at either 24°C or 36°C for 8 hours. During this period, the viability of the cells was monitored by diluting and transferring the cells to solid YEPD media and allowing the plates to incubate at 24°C for 6 days. No loss of viability was detected in this diploid strain.

Growth of Strain TS6M4.6 in YEPD Media

A culture of TS6M4.6 grown at 24°C in liquid YEPD media was diluted into YEPD media at a concentration of 1.3×10^7 cells per ml. Equal aliquots of the diluted culture were then incubated at 24°C or 36°C for 8.5 hours. During this period, the growth of the cells was monitored by 1) changes in turbidity of the culture and by 2) diluting and transferring aliquots of the culture onto solid YEPD media (see Materials and Methods). As can be seen in Figure 15, cells incubated at 36°C exhibit a rapid reduction of viability, while the corresponding growth and viability of the cells incubated at 24°C appears normal. Comparison of this result to that obtained when growth was monitored by changes in optical density, as measured by a Klett-Summerson colorimeter, can be seen in Figure 16. Here it can be noted that during the

initial 6 hours after the shift to the elevated temperature, the rate of optical density increase in the culture incubated at 36°C is identical to that of the parallel culture incubated at 24°C. This continued increase in turbidity while the culture is rapidly losing viability is very similar to that observed with several of the cell division cycle mutants (cdc) isolated by Hartwell (Hartwell, 1967). However, unlike the cdc mutants which produce yeast cells with aberrant morphologies, the TS6M4.6 cells appear structurally normal even after 10 hours of incubation at 36°C.

In order to determine the degree to which the TS6M4.6 mutant gene product is sensitive to increased temperatures, a culture of this strain was diluted into 12 ml of fresh YEPD media at a concentration of 2.25×10^7 cells per ml and then divided into three equal aliquots. These cultures were then incubated at either 24°C, 30°C or 36°C for 3 hours and the number of viable cells in each determined at hourly intervals by diluting and plating aliquots of the experimental cultures onto YEPD media. These plates were then incubated for 6 days at 24°C and the number of cells able to form colonies determined. Figure 17 summarizes the results of this experiment in which it can be seen that the TS6M4.6 mutant gene product expresses temperature sensitivity even at the intermediate temperature of 30°C.

Phenotypic Expression of the TS6M4.6 Mutation in Cells Unable to Grow and Divide

In Figures 15 and 16, it can be seen that during the initial 2 hours after the transfer of strain TS6M4.6 to the restrictive temperature

there exists a correspondence between the lag in growth, as measured by optical density determinations, and a slower rate of cell death. This observation suggested that the expression of temperature sensitive lethality in this mutant may, perhaps, be dependent on the continuing ability of the cells to grow. This prompted the following experiment in which a culture of TS6M4.6 grown to mid log phase at 24°C in liquid YEPD media was centrifuged and resuspended in YNBD media lacking the auxotrophic supplements histidine and uracil upon which the cells are dependent for growth (see genotype in Table 1). This culture was then allowed to incubate at 24°C for a period of 6 hours, after which the starved cells were transferred to the restrictive temperature and the number of viable cells determined for a further 7 hour period. As can be seen in Figure 18, the number of viable cells remained constant during this experimental 7 hour exposure to 36°C, thus suggesting that the expression of this mutation was indeed dependent on the continued growth of the cells.

DNA Metabolism in Strain TS6M4.6

Several of the cdc lesions have been shown to exhibit blockages in one or another of the DNA metabolism processes (Hartwell, 1973). In order to determine whether the phenotype of TS6M4.6 is similarly due to aberrant DNA metabolism, a series of experiments were performed.

1) Incorporation of a DNA precursor into hot alkali stable cold

acid precipitable material at the permissive and non-permissive temperature.

A 40 ml culture of strain TS6M4.6 grown at 24°C to mid log phase in liquid YNBD + X media was divided into two equal aliquots which were then incubated at either 24°C or 36°C in the presence of 9 µg per ml cold adenine and 10 µCi per ml ^{3}H-adenine. At houly intervals, for a period of 5 hours, three 0.5 ml samples were removed from each of the experimental cultures and the amount of ^{3}H-adenine incorporated into the DNA as measured by hot alkali stable cold acid precipitable counts, analyzed as described in the Materials and Methods. As can be seen in Figure 19, the amount of incorporation of radioactive adenine into the DNA at the restrictive temperature of 36°C is nearly identical with that of the 24°C control. Thus, the loss of viability expressed in this strain at the restricitve temperature is not the result of a temperature sensitive block in the DNA synthetic apparatus.

2) The fidelity of the DNA synthesized at the permissive and non-permissive temperatures by strain TS6M4.6.

Following the observation that the rate of DNA synthesis appears to be taking place normally at the restrictive temperature, the question arose as to whether the observed synthesis taking place at the restrictive temperature is accurate with regards to genetic fidelity. It was postulated that errors could be accumulating during DNA synthesis at the non-permissive temperature and that these errors result in the loss of

viability in this mutant.

a) Search for mutational induction of additional auxotrophic requirements at the non-permissive temperature. A culture of TS6M4.6, pregrown in liquid YEPD to mid log phase at 24°C, was shifted to the restrictive temperature and the cells transferred at hourly intervals for a period of 6 hours to solid YEPD media. The surviving cells were allowed to grow into colonies at 24°C and were then analyzed by replica-plating onto dropout media on which only the parental genotype could survive (YNBD + UR + HIS). If the temperature sensitive mutation had expressed itself in these survivors by creating additional auxotrophy, then some of these colonies would not be expected to grow on the YNBD + UR + HIS media. This was not found to be the case and all 758 survivors could grow on both types of media. This absence of any additional auxotrophic requirements in the surviving colonies can be interpreted in two ways; either that the temperature sensitive lethality expressed in this strain is not the result of a mutagenic event, or that the mutagenic event produced in the absence of the TS6M4.6 gene product is too severe to allow cells with only one complement of the genome to survive. Therefore, a comparable experiment to the one described above was performed on a prototrophic diploid strain (x459), homozygous for the temperature sensitive lesion and heterozygous for various auxotrophic markers (see Table 1). In such a diploid, a mutagenic event on the wild type homologue of any of the several heterozygous auxotrophic loci

could yield an auxotrophic diploid, while it is possible that a recessive mutagenic event, which would normally have been lethal in the haploid, would not be lethal in this diploid because of the complementary copy of the genome. None of the 219 colonies that survived varying lengths of exposure to 36°C, when replicaplated onto YNBD media, a media which supports the growth of strain x459, showed the need for any additional auxotrophic supplements.

b) Rescue of potentially lethal events by mating. In this second approach undertaken to determine if the inviability expressed by TS6M4.6 at 36°C is due to a lethal mutagenic event, a 30 ml culture of TS6M4.6 grown in liquid YEPD to mid log phase was divided into six 5 ml aliquots which were exposed to the restrictive temperature for varying periods of time (0 to 5 hours). Each experimental culture was then returned to the permissive temperature, mixed with 5 ml ($\sim 10^7$ cells/ml) of a non-temperature sensitive "a" mating type strain (A364A: for genotype, see Table 1) and allowed to mate for 6 hours at 24°C. These mating mixtures were then transferred onto solid media selective for the growth of any resultant diploids (YNBD + HIS). The diploid cells produced in this mating were allowed to form colonies at 24°C. These colonies were then induced to undergo meiosis on KAc media, also at 24°C, and micromanipulation of the meiotic products performed. As can be seen in Figure 20, increasing the time of exposure of TS6M4.6 to the restrictive temperature prior to mating, results in a decrease

in the total nunber of viable spores produced from these diploids. This decrease in spore viability manifests itself in a rapid decrease in the number of tetrads yielding 4 viable spores in conjunction with a rapid increase in the number of tetrads yielding 2, 3, or 4 inviable spores (see Figure 21).

Genetic analysis of the completely viable tetrads show normal patterns of marker segregation (see Table 2) and are believed to be the result of a mating involving a TS6M4.6 cell which has as yet not been affected by the elevated temperature. However, genetic analysis of the spores obtained from the incompletely viable tetrads show unusual patterns of genetic segregation in which the ratios of the genetic markers appear extremely aberrant (see Table 2). The extremely high frequency amongst the surviving spores of the lysine, tyrosine and adenine prototrophic phenotypes suggested the possibility that some of these spores might be aneuploid containing both the "+" and "-" alleles for these genetic markers. Furthermore, analysis of the mating types of the surviving spores (see Materials and Methods) revealed that some of these spores were of a sterile nature (see Table 3), mating at a low frequency to both a and α mating types tester strains (Pomper et al., 1954). Four of these sterile spores, when transferred to KAc media at the permissive temperature, were found to sporulate after five days, thus suggesting the presence in some of these surviving spores of genetic information for both a and α mating types (see Discussion).

Two surviving spores (BR.4.7B - ura_3his_7 and BR.4.8B - ura_3his_7),

derived from diploids created in a cross between TS6M4.6 exposed to 36°C for three hours and strain A364A, expressing the prototrophic phenotype for lysine, tyrosine and adenine were mated to a non-temperature sensitive laboratory stock strain (101.7A - a ura_1). The resulting diploids isolated on selective media were induced to undergo sporulation and the resulting meiotic products analyzed. The diploids derived from the mating of the BR4.7B spore produced spores that exhibited auxotrophy for the lysine, tyrosine and adenine genetic markers, while the diploids derived from the mating of the BR4.8B spore produced normal segregation patterns (see Table 4). The expression of auxotrophy for the lysine, tryosine and adenine demonstrates that the BR.4.7B spore must contain hidden "-" alleles for these genetic markers. Furthermore, when allele testing was performed on the spores created in these two crosses (BR.4.7B x 101.7A and BR.4.8B x 101.7A) in order to determine the segregation pattern of the uracil auxotrophic alleles, it was noted that in the BR.4.7B cross only one spore expressed the ura_1^- genotype while in the BR.4.8B cross the expected 2:2 ratio of segregation of the ura_1^- marker was observed (see Table 5). These results suggest the existence in some of the surviving spores of an excess of the ura_1^+ genetic information.

In a control to the experiment described above, three conditional mutants (cdc 4,7, and 8) defective in specific steps in the cell cycle were exposed to the elevated temperature for 0, 3, and 6 hours. These cells were mated at the permissive temperature to strain 123.1C and

e diploids isolated as before were then made to undergo meiosis at 24°C. Figure 22 demonstrated that unlike TS6M4.6, there is no loss of spore viability associated with the exposure of these cells to the restrictive temperature.

DISCUSSION

Chapter 3. Analysis of the Mutant Conditionally Deficient in Mitochondrial DNA Metabolism

A mutant is described in this communication which conditionally affects mitochondrial DNA metabolism and results in the production of petites at the restrictive temperature. Tetrad analysis demonstrates that the temperature sensitive phenotype of this mutant is a result of a single nuclear gene mutation. This mutant gene appears to be of a recessive nature, for in an experiment in which the mutant strain was mated to two different laboratory stock strains and the resulting diploids, heterozygous for the petite inducing mutation analyzed, it was found that respiratory competent diploid cells continue to be produced even when grown at the elevated temperature.

Cells growing at the restrictive temperature in media containing dextrose as its sole carbon source produce petites which are neutral and appear to be devoid of mitochondrial DNA. The DNA synthesis pattern of the mutant strain growing at the restrictive temperature, in dextrose media, shows a reduction in the incorporation of radioactive adenine into the mitochondrial genome during the initial four hours after the transfer to the elevated temperature. These results suggest that either: i) mitochondrial DNA synthesis is absent or possibly reduced at the restrictive temperature, or ii) mitochondrial DNA is degraded at the restrictive temperature. When a culture of cells labeled at the permissive temperature with radioactive adenine was transferred to the restrictive temperature, the prelabeled mitochondrial DNA was found to be stable for at least 4 hours, thus

suggesting that the phenotype in dextrose media is a result of the absence or reduction of mitochondrial DNA synthesis and not due to the degradation of the mitochondrial DNA.

Mutant cells growing at the restrictive temperature in media containing glycerol as its sole carbon source, induce petites which are suppressive and contain mitochondrial DNA. Mitochondrial DNA synthesis of the mutant strain grown at the restrictive temperature in glycerol media occurs at a level comparable to that of the control for the initial 4 hours after the transfer to the elevated temperature.

We thus appear to have isolated a mutant that expresses different phenotypes when grown under different growth conditions. Growth of this strain in media containing dextrose as its sole carbon source produces petites which are of a neutral nature and appear to be devoid of mitochondrial DNA. However, growth of this strain at the restrictive temperature in media containing glycerol as its sole carbon source produces petites which are suppressive and contain mitochondrial DNA.

By varying the carbon source of the growth medium, yeast mitochondria can be made to undergo repression and derepression. This repression and derepression is accompanied by many physiological changes that include permutations in the structure of the mitochondria (Yotsuyanagi, 1962a), the amounts of mitochondrial DNA as compared to the nuclear species (Cottrell and Avers, 1970), the transmission of mitochondrial markers to progeny (Goldthwaite _et al._,

1974), and the rate at which some mitochondrial membrane proteins are synthesized (Mian *et al.*, 1973). We suspected that the different phenotypes expressed by this mutant in media containing dextrose or glycerol might be due to the repressive and derepressive nature of these carbon sources. In a recent report (Hall *et al.*, 1975) evidence was presented that demonstrated a functional association of mitochondrial DNA replication with the mitochondrial membrane in *S. cerevisiae*. It was therefore suggested that when the mitochondria of the mutant cells are in a repressed state, the mitochondrial membranes are altered such that at the restrictive temperature the mutation will not allow the mitochondrial DNA to be replicated. On the other hand, when the mitochondria of the mutant cells are in a non-repressed state, at the restrictive temperature, the mitochondrial membranes permit mitochondrial DNA synthesis to take place, however, the synthesis occurs incorrectly.

In order to determine if the different phenotypes of the mutant were in fact the result of the repressed versus the derepressed state of the mitochondria, experiments were performed in order to determine what the phenotypic expression of this temperature sensitive mutation is in media containing galactose, which though fermentable, is believed to be of a non-repressing nature (Tustanoff and Bartley, 1964).

Cells grown at the restrictive temperature in media containing galactose as its sole carbon source were found to induce petites which are of a neutral nature and appear to be devoid of mitochondrial DNA.

Such a phenotypic expression of this mutation in galactose containing media is similar to that observed when cells are grown in the repressing dextrose media. Thus, the different expressions of this mutation on repressing and non-repressing carbon sources may indeed not be the result of permutations brought about in the mitochondria by the repressing and non-repressing natures of dextrose, glycerol and galactose. This, of course, would only be true if galactose is indeed a non-repressing carbon source.

Kinetic analysis of the turn-off of mitochondrial DNA synthesis by this mutant strain has revealed that cells grown at the restrictive temperature in dextrose or glycerol containing media exhibit a greatly reduced amount of incorporation of radioactive adenine into the mitochondrial DNA after two hours of exposure to the elevated temperature. These results suggest the presence, in this mutant, of a temperature sensitive block in the mitochondrial DNA synthetic apparatus. This reduced incorporation of radioactive adenine in dextrose grown cells after the transfer to the elevated temperature was previously observed in the experiment in which mitochondrial DNA synthesis was monitored, by radioactively labeling cells, for a four hour period following the transfer of the culture to the elevated temperature. However, the reduction of incorporation into the mitochondrial DNA of glycerol grown cells was not previously observed. These apparently contradictory results may perhaps be due to the increased amounts of mitochondrial DNA initially present in these cells under

non-repressing conditions (Cottrell and Avers, 1970). The presence of a greater amount of mitochondrial DNA in glycerol grown cells may enable the incorporation of radioactive adenine into the mitochondrial DNA to occur over a short time interval and thus, in glycerol media, the incorporation of label into the mitochondrial DNA during the first four hours at the restrictive temperature could be taking place before the conditional block occurs and , therefore, does not reflect any temperature sensitivity.

We thus appear to have isolated a conditional mutant of *Saccharomyces cerevisiae* which, as one of its properties, exhibits a temperature sensitive block in its mitochondrial DNA synthetic apparatus. If, however, the production of petites in this strain when grown at the restrictive temperature is solely the result of a temperature sensitive block in the mitochondrial DNA metabolism, then all petites induced by this strain at the elevated temperature would be expected to lack mitochondrial DNA. However, this has not been found to be the case, as growth of the mutant strain at the elevated temperature in glycerol media has yielded petites which do indeed contain mitochondrial DNA. This result suggests that in addition to an apparent block in mitochondrial DNA synthesis there is also, at least in glycerol media and perhaps in dextrose media as well, a mutagenic event occurring which results in the production of petites. The inability to isolate petite strains containing mitochondrial DNA from cells grown at the restrictive temperature in dextrose media may perhaps be due to the fact that in the petite

state such cells can continue to grow on the dextrose media, and in the absence of mitochondrial DNA synthesis, "dilute out" their mitochondrial DNA so that most of the petites isolated lack any detectable quantities of mitochondrial DNA. However, in glycerol media the petite cells induced by the mutagenic event are unable to "dilute out" their mitochondrial DNA, thus enabling the isolation of petite cells containing mitochondrial DNA.

Finally, it has been reported that various treatments such as anaerobosis (Pinto et al., 1975), glucose repression (Hollenberg and Borst, 1971) and nalidixic acid treatment (Vidova and Kovac, 1972) are able to reduce the mutagenic effect of ethidium bromide in producing petites. The observation that anaerobic growth and glucose repression result in the disappearance of some mitochondrial enzymes and in modifications of mitochondrial membranes (Criddle and Schatz, 1969) suggested an experiment in which the expression of the petite inducing phenotype of this mutant strain be analyzed in a respiratory incompetent cell in order to determine if a respiratory incompetent state might effect the expression of this temperature sensitive mutation. A suppressive petite strain containing mitochondrial DNA and harboring the temperature sensitive lesion was grown at the restrictive temperature in dextrose containing media. This strain was observed to produce neutral petites which lacked mitochondrial DNA. Thus it appears that the expression of this petite inducing phenotype is not dependent on the respiratory capacity of the mutant cell.

Chapter 4. Analysis of the Mutant Conditionally Deficient in Nuclear DNA Metabolism

A mutant of *Saccharomyces cerevisiae* is described in this communication which conditionally affects nuclear DNA metabolism. Tetrad analysis demonstates that the temperature sensitive phenotype of this mutant is the result of a single nuclear gene mutation.

When a culture of this mutant strain is transferred to the restrictive temperature a continued increase in optical density occurs which is accompanied by a rapid loss of cell viability. This loss of cell viability appears to require the continued growth of the mutant cells, since cells that have been starved prior to and during exposure to the restrictive temperature fail to exhibit any loss of cell viability even after seven hours.

The ability of the mutant to synthesize DNA at the restrictive temperature was monitored in order to determine whether or not the loss of viability is the result of an inability of these cells to synthesize DNA at the elevated temperature. The rate of DNA synthesis at the elevated temperature, as measured by incorporation of radioactive adenine into hot alkali stable cold acid precipitable material, occurs at a rate comparable to that of the 24°C control. Thus, unlike the cdc mutants, (Hartwell, 1973) the loss of viability expressed in this strain is not the result of a temperature sensitive block in the DNA synthetic apparatus.

The accuracy with which this mutant was able to maintain correct DNA synthesis at the non-permissive temperature, was analyzed in the following manner. Cells of the mutant strain were exposed to the elevated temperature for varying lengths of time and then returned

to the permissive temperature. Subsequently, all manipulations and procedures, including mating with a non-temperature sensitive strain, selection of the diploids, sporulation, micromanipulation, and tetrad analysis were all carried out at the permissive temperature. A total elapsed time of often up to twelve to fifteen days had therefore passed between initial exposure of the mutant haploid to the restrictive temperature and the subsequent analysis of the micromanipulated spores. The first observation was that the number of viable spores produced from these diploids was greatly reduced. Thus exposure of the haploid mutant cell to the restrictive temperature appears to induce a permanent alteration which is maintained even during the subsequent long incubation at the permissive temperature.

By comparison, when an identical experiment to the one described above was performed using several temperature sensitive cdc mutants, there was no reduction in the number of viable spores produced by the resulting diploids. Thus, it would appear that the loss of spore viability in the TS6M4.6 crosses is not the result of the "unhealthy" nature of one of the mating partners.

Secondly, examination of Figure 21 indicates that with increasing time of exposure of the mutant haploid cell to 36°C, not only does the frequency with which an ascus containing inviable spores increase, but this expression is not a completely random process, as can be seen. After the second hour, the number of tetrads with four viable spores declines rapidly yielding no four viable spored tetrads after five hours of exposure. This result is accompanied by a concomittant rise in

the number of tetrads yielding 2 or 0 viable spores per ascus. At a later time (3 to 5 hours) the number of asci having only one viable spore also increases. However, at no time does a significant number of tetrads yield three viable spores per ascus. This type of spore survival pattern would be expected if one or more lethal genetic aberrations were occurring in the mutant during the induction period and were then segregating during the meiotic process.

Thirdly, an analysis of the genetic segregation pattern of the genetic markers in the surviving spores has demonstrated extreme deviations from expected values. There exists among these surviving meiotic products, a preferential transmission of the "+" alleles for the lysine, tyrosine and adenine auxotrophic markers. It is interesting to note that these "+" alleles were originally introduced into the diploids by the temperature sensitive parent, thus suggesting that there may perhaps be a preferential replication in the temperature sensitive parent, at 36°C, of some of its genetic information.

Fourthly, analysis of the mating type of the surviving spores revealed that 24% are sterile. In 1943, Lindegren and Lindegren demonstrated that a single pair of alleles "a" and "α", control the mating response in *Saccharomyces cerevisiae*. Subsequently, it was demonstrated that the "a/α" diploids behaved in a sterile fashion, mating only rarely with either "a" or "α" mating type strains (Pomper *et al.*, 1954). The sterile spores isolated from the tetrads yielding less than four viable spores were analyzed to determine if their sterile

phenotype is the result of such a heterozygosity of the mating locus. In this analysis, advantage was taken of the fact that anueploid strains containing genetic information for both the "a" and "α" mating types have the ability to initiate some of the processes involved in sporulation (Roth and Fogel, 1971). Thus, the sterile spores were transferred to KAc sporulation media and scored for their ability to undergo partial or complete sporulation. This analysis revealed that several of these sterile spores did in fact have the ability to undergo sporulation. The results presented suggest (see Table 3) that 78% of the surviving spores contain "α" mating type information (α - 54%; a/α - 24%) while 46% of the surviving spores contain "a" mating type information. Thus, once again, there appears to be evidence to suggest that there may be a preferential transmission of some (or all) of the genetic information of the temperature sensitive parent at the elevated temperature.

Finally, when two of the surviving spores were analyzed genetically, one was found to contain both "+" and "-" alleles for the lysine, tyrosine and adenine genetic markers, as well as an excess of the prototrophic allele for the ura_1 locus. Thus, these data demonstrate the existence of aneuploidy or at least multiple copies of certain alleles in the surviving spores.

All of the above findings are consistant with the general hypothesis that at the non-permissive temperature inaccurate replication of the mutant cell's genome is occurring. While it appears that DNA synthesis

continues to take place, subsequent analysis of the material synthesized has shown it to be incorrect. This can be seen initially by the dramatic loss of cell viability and can be studied further if these damaged cells are rescued by mating and then allowed to segregate their genetic material during the process of meiosis. Some of the genetic material in these meiotic products can no longer be detected (i.e. loss of spore viability) while extra genetic material appears to have accumulated (i.e. aberrant segregation patterns of various genetic markers). The extended period of incubation at the permissive temperature between the time of exposure to the elevated temperature and induction of meiosis, rules out the possibility that the expression of this mutation manifests itself in an alteration of a cellular component that is turned over, and strongly suggests that a permanent genetic alteration is being induced in this strain.

The data presented here suggests that the absence of the wild type TS6M4.6 nuclear gene product at the elevated temperature in this temperature sensitive strain results in a loss of the cells' control of either the replication, maintenance or distribution of chromosomal information. This defect manifests intself in the haploid by producing cells which, after exposure to the restrictive temperature, and perhaps due to a loss of genetic information, have lost their colony forming ability. This loss of chromosomal control further expresses itself when cells which had been exposed to the restrictive temperature were rescued by a mating to a non-temperature sensitive strain at the permissive temperature. These resulting diploids, when made to undergo meiosis, produced numerous inviable spores,

as well as viable spores exhibiting extremely aberrant genetic marker segregations due to anueploidy of some or all of the chromosomes (or alleles). These results suggest that in addition to a possible loss of genetic information due to the absence of the TS6M4.6 gene product there may also be an accumulation of genetic information in this mutant strain at the elevated temperature.

Table 1

Strains of Saccharomyces cerevisiae

Strain number	Ploidy	Genotype
123.1C	1n	α $ura_3 his_7 gal^-$
123.1C Gal^+	1n	α $ura_3 his_7 gal^+$
TS-1G-95b	1n	α $ura_3 his_7 tpi$
CR-9B	1n	a $lys_2 leu_2$
144-2C	1n	a $leu_2 ura_3 his_7 gal^- tpi$
144-2C Gal^+	1n	a $leu_2 ura_3 his_7 gal^+ tpi$
144-2C ρ^--3	1n	a $leu_2 ura_3 his_7 gal^- tpi$ ρ^-
103.1A	1n	α his_1
115.8C	1n	α lys_2
103.1D	1n	α lys_1
x463 (144-2C x 115.8C)	2n	$\frac{a\ leu_2\ ura_3\ his_7\ +\ tpi}{\alpha\ +\ +\ +\ lys_2\ +}$
x464 (144-2C x 103.1D)	2n	$\frac{a\ leu_2\ ura_3\ his_7\ +\ tpi}{\alpha\ +\ +\ +\ lys_1\ +}$
TS6M4.6	1n	α $ura_3 his_7 tsl$
102.10A	1n	a lys_2
A364A	1n	a $ade_1 ade_2 his_7 ura_1 lys_2 tyr_1$
TS314	1n	Isogenic with A364A; also contains cdc 4-1

Table 1 (cont.)

Strains of Saccharomyces cerevisiae

Strain number	Ploidy	Genotype
TS124	1n	Isogenic with A364A; also contains cdc 7-1
TS198	1n	Isogenic with A364A; also contains cdc 8-1
x403 (TS6M4.6 x 102.10A)	2n	$\frac{\alpha\ ura_3 his_7\ +\ tsl}{a\ +\ +\ lys_2\ +}$
457.5D	1n	α $ade_1 his_7 ura_3 lys_2 tyr1$ tsl
458.2D	1n	a lys_1tsl
x459 (457.5D x 458.2D)	2n	$\frac{\alpha ade_1 his_7 ura_3 lys_2 tyr_1\ +\ tsl}{a\ +\ +\ +\ +\ +\ lys_1 tsl}$
101.7A	1n	a ura_1

tpi - temperature sensitive petite inducer (Rubin and Blamire, 1977)
tsl - temperature sensitive lethal

Table 2

Auxotrophic Phenotype of Spores Produced From Diploids Created in a Cross Between Cells of TS6M4.6 Exposed to 36°C for Varying Lengths of Time Prior to Mating and Strain A364A

	Total no. of Spores	HIS		URA		LYS		ADE		TYR	
		+	-	+	-	+	-	+	-	+	-
Complete Tetrads	108	0	108	26	82	55	53	20	88	55	53
Incomplete Tetrads	41**	0	41	12	29	40	1	21	20	37	4
Expected Ratios		0	1	1	3	2	2	1	3	2	2
P*(Complete Tetrads)		1.00		.80-.90		.80-.90		.10-.20		.80-.90	
P*(Incomplete Tetrads)		1.00		.50-.70		<.01		<.01		<.01	

*- Probability calculated according to Chi square method.

** These 41 spores were obtained from the dissection of 24 four spored asci.

Table 3

Mating Type of Spores Produced From Diploids Created in a Cross Between Cells of TS6M4.6 Exposed to 36°C for Varying Lengths of Time Prior to Mating and Strain A364A

	Total no. of spores	a	α	Sterile
Complete Tetrads	108	53 (49%)	54 (50%)	1 (1%)
Incomplete Tetrads	41	9 (22%)	22 (54%)	10 (24%)
Expected Ratios		2	2	0
P (Complete Tetrads)		> .90		
P (Incomplete Tetrads)		.05 - .10		

Table 4

Auxotrophic Phenotype of Spores Produced From Diploids Created in a Cross Between Cells of BR.4.7B and 101.7A and in a Cross Between Cells of BR.4.8B and 101.7A

	HIS		URA		LYS		AD		TYR	
	+	-	+	-	+	-	+	-	+	-
BR.4.7B x 101.7A	7	10	7	10	13	4	14	3	14	3
BR.4.8B x 101.7A	22	22	8	36	44	0	44	0	44	0
Expected Ratios	1	1	1	3	1	0	1	0	1	0
P (BR.4.7B x 101.7A)	.30-.50		.10-.20		<.01		<.01		<.01	
P (BR.4.8B x 101.7A)	1.00		.20-.30		1.00		1.00		1.00	

Table 5

Observed Uracil Genotype of Spores Produced From Diploids Created in a Cross Between Cells of BR.4.7B and 101.7A and in a Cross Between Cells of BR.4.8B and 101.7A

	URA_1^+	URA_1^-	URA_3^+	URA_3^-
BR.4.7B x 101.7A	16	1	8	9
BR.4.8B x 101.7A	22	22	22	22
Expected Ratios	2	2	2	2
P (BR.4.7B x 101.7A)	$<.01$		.80 - .90	
P (BR.4.8B x 101.7A)	1.00		1.00	

Figure 1. Petite induction of strain x463 at 24°C (o—o) and 36°C (●—●) and strain x464 at 24°C (o- - -o) and 36°C (●- - -●). Cells of strains x463 and x464 growing in liquid YEPD media at 24°C were diluted into 10 ml aliquots of fresh YEPD at a concentration of 1.57×10^3 and 1.4×10^3 cells/ml respectively and allowed to grow for approximately 15 generations at either 24°C or 36°C. The induction of petites was monitored during this period by diluting and plating cells from both temperatures onto solid YEP-Dif media and allowing the cells to grow for 6 days at 24°C. The number of grandes remaining were scored on the basis of colony size and recorded as a percentage of the total population.

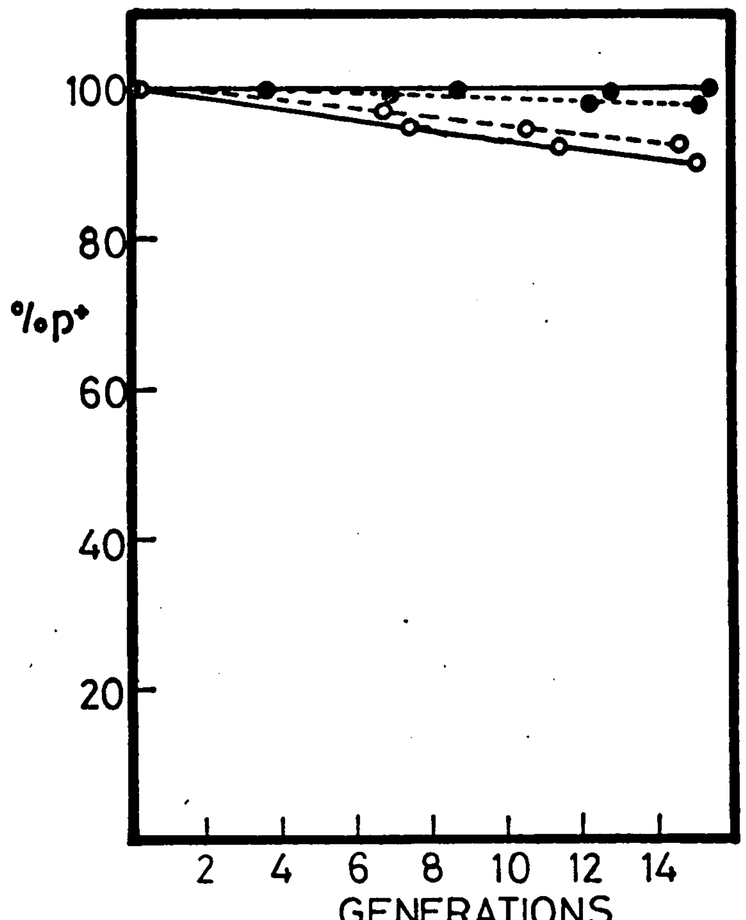

100
80
60
40
20
%p+
2 4 6 8 10 12 14
GENERATIONS

Figure 2. Petite induction of strain 144-2C grown at 24°C (o—o) and 36°C (●—●) in dextrose media. Cells growing in liquid YEPD at 24°C were diluted into 10 ml aliquots of YEPD media to give a final concentration of 5×10^3 cells/ml, and allowed to grow at either 24°C or 36°C for 6 generations. The induction of petites was monitored during this period as described in the legend to Figure 1.

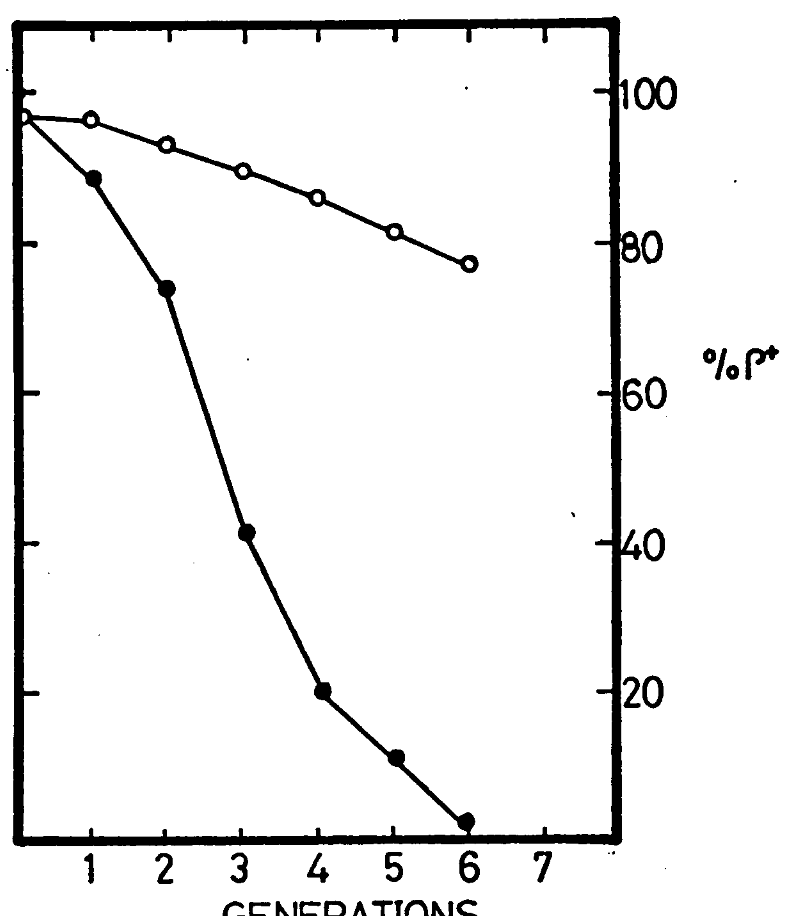
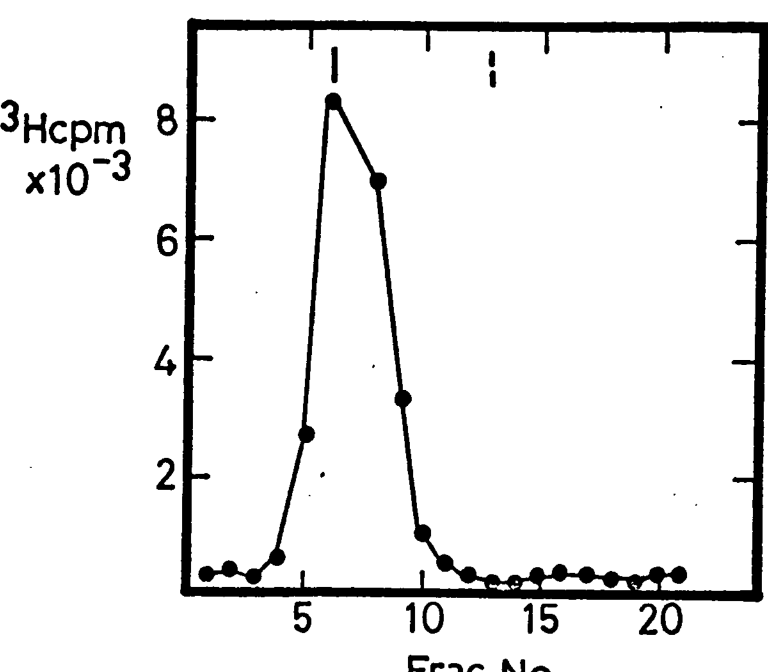

100
80
60
40
20
%ρ+
1
2
3
4
5
6
7
GENERATIONS

Figure 3. Cesium chloride density gradient DNA profile of a ^{3}H-adenine labeled petite clone isolated from strain 144-2C after exposure to 36°C in dextrose media. The 4 hour labeling of this petite strain with 20 μCi/ml of ^{3}H-adenine in YNBD + X media was carried out in the presence of 200 μg/ml of cycloheximide in order to increase the incorporation of label into the mitochondrial genome. After the 4 hour incubation period the cells were harvested, lysed, and the lysates centrifuged for 42 hours at 33,000 rpm at 19°C in a Spinco 50 Ti rotor. In this gradient, as well as all others, ^{14}C-adenine labeled DNA of the grande strain 123.1C was included as a marker (not shown). Samples, 0.2 ml, were collected from the bottom of the gradient and assayed for hot alkali stable cold TCA precipitable counts. The solid vertical line and the dashed vertical line indicate the respective positions of the ^{14}C-adenine labeled nuclear and mitochondrial DNA species of parental strain 123.1C.



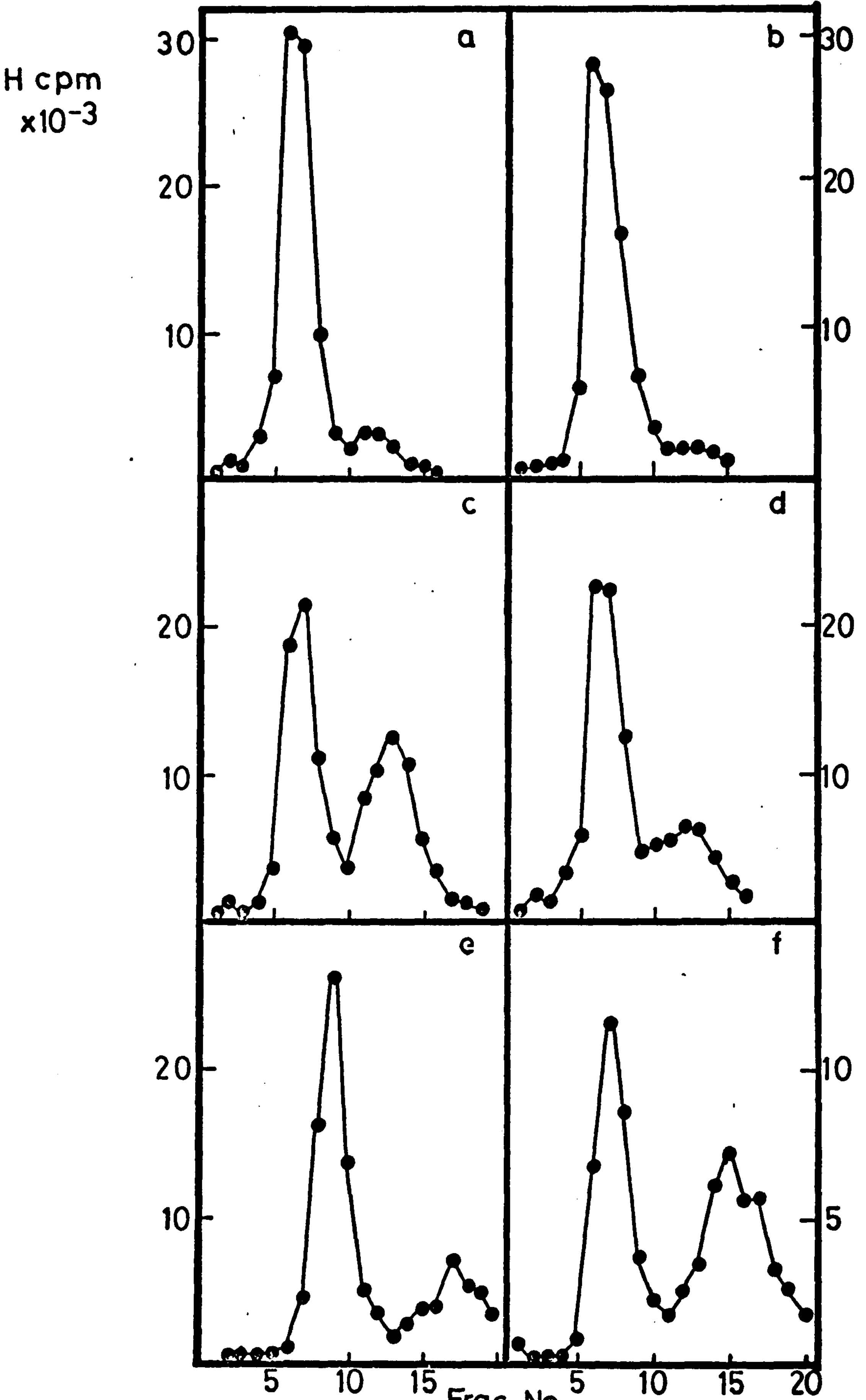
3Hcpm x10-3
8
6
4
2
5
10
15
20
Frac.No.

Figure 4a-f. CsCl density gradient DNA profiles of cells grown in YNBD + X media in the presence of 20 μCi/ml ^{3}H-adenine for 4 hours.

(a & b) Radioactive incorporation of strain 144-2C at 24°C (a) and 36°C (b).

(c & d) Radioactive incorporation of strain 144-2C in the presence of 200 μg/ml of cycloheximide at 24°C (c) and 36°C (d).

(e & f) Radioactive incorporation of parental strain 123.1C at 24°C (e) and 36°C (f).



^{3}H cpm
x10^{-3}
a
b
c
d
e
f
30
20
10
5
15
Frac. No.

Figure 5a-b. CsCl density gradient DNA profiles of strain 144-2C, prelabeled for 15 hours at 24°C in the presence of 20 µCi/ml of ^{3}H-adenine in liquid YNBD + X. These cells were then washed, resuspended in fresh non-radioactive media, and divided into two equal aliquots which were then incubated at 24°C (a) and 36°C (b) for 4 hours.

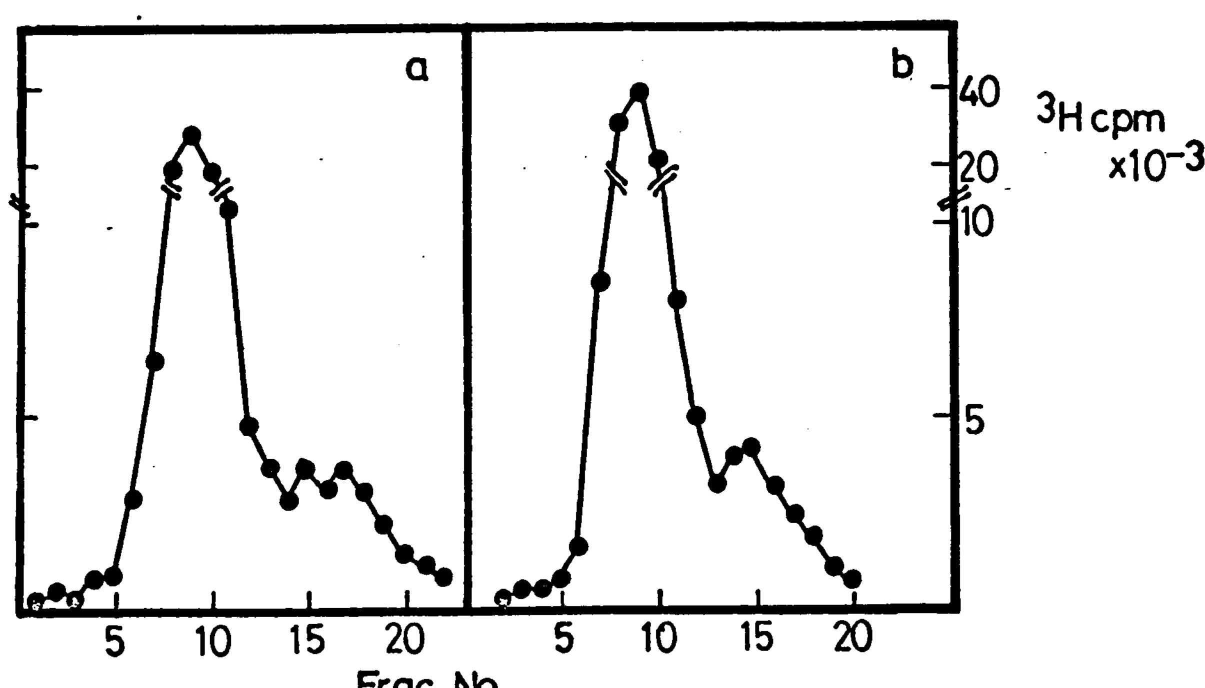
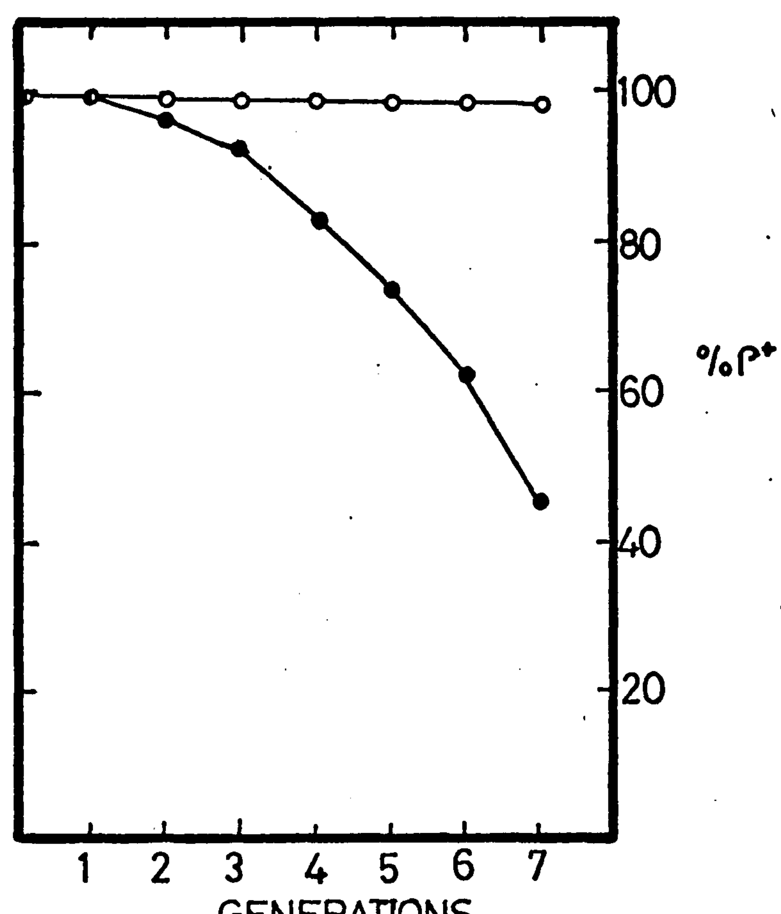

a
b
40
20
10
5
^{3}H cpm
x10^{-3}
5
10
15
20
5
10
15
20
Frac. No.

Figure 6. Petite induction of strain 144-2C grown at $24^{\circ}C$ (o—o) and $36^{\circ}C$ (●—●) in glycerol media. Cells growing in liquid YEPG media at $24^{\circ}C$ were diluted in YEPG media and allowed to grow at either $24^{\circ}C$ or $36^{\circ}C$ for 7 generations. Petite induction during the period was monitored by diluting and plating onto solid YEP-Dif media as described in the legend to Figure 1.



100
80
60
40
20
%ρ+
1 2 3 4 5 6 7
GENERATIONS

Figure 7. CsCl density gradient DNA profile of a ^{3}H-adenine labeled petite clone isolated from strain 144-2C after exposure to 36°C in glycerol media. The labeling of the petite strain in YNBD + X media was carried out using 20 µCi/ml ^{3}H-adenine in the presence of 200 µg/ml of cycloheximide. The cells were then harvested, lysed, and analyzed as described in the legend to Figure 3.

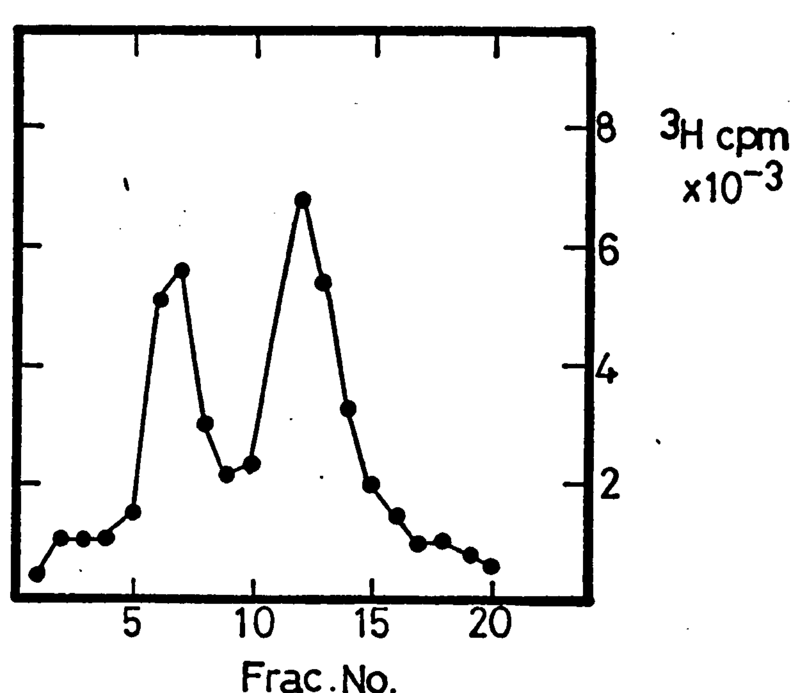

8
6
4
2
^{3}H cpm
x10^{-3}
5
10
15
20
Frac.No.

Figure 8a-d. CsCl density gradient DNA profiles of cells grown in YNBG + X media in the presence of 20 µCi/ml of ^{3}H-adenine for 4 hours.

(a & b) Radioactive incorporation of strain 144-2C at 24°C (a) and 36°C (b).

(c & d) Radioactive incorporation of parental strain 123.1C at 24°C (c) and 36°C (d).

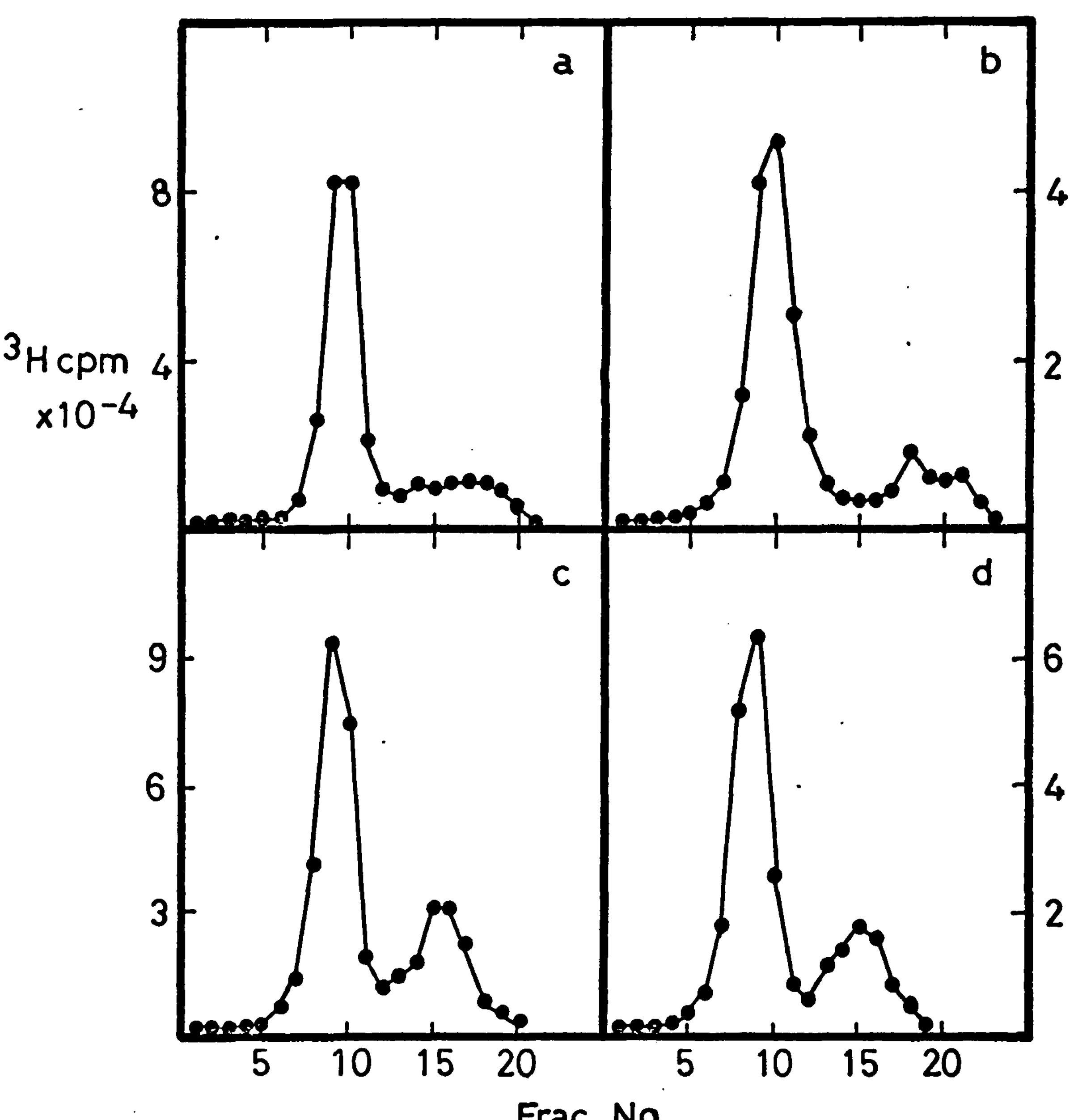

a
b
c
d
^{3}H cpm
x10^{-4}
8
4
4
2
9
6
3
6
4
2
5
10
15
20
5
10
15
20
Frac. No.

Figure 9. Petite induction of strain 144-2C Gal^{+} at 24°C (o—o) and 36°C (●—●) in galactose media. Cells growing in liquid YEP-Gal at 24°C were diluted into 10 ml aliquots of fresh YEP-Gal media at a concentration of 3.2×10^{4} cells/ml and allowed to grow for approximately 7 generations at 24°C or 36°C. Petite induction during this period was monitored as described in the legend to Figure 1.

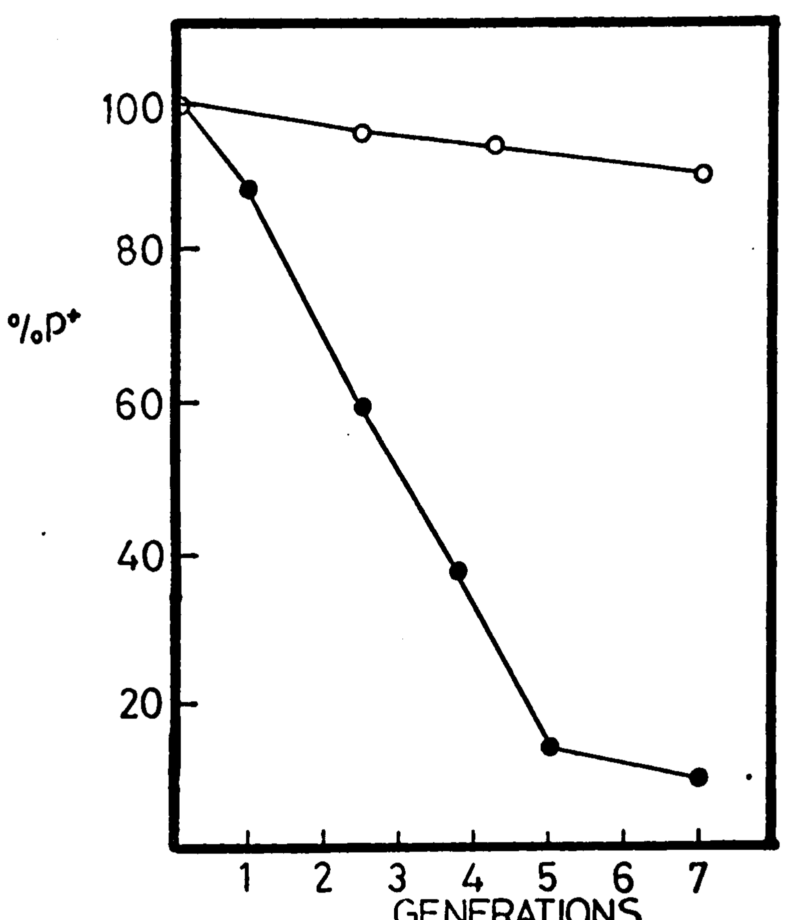
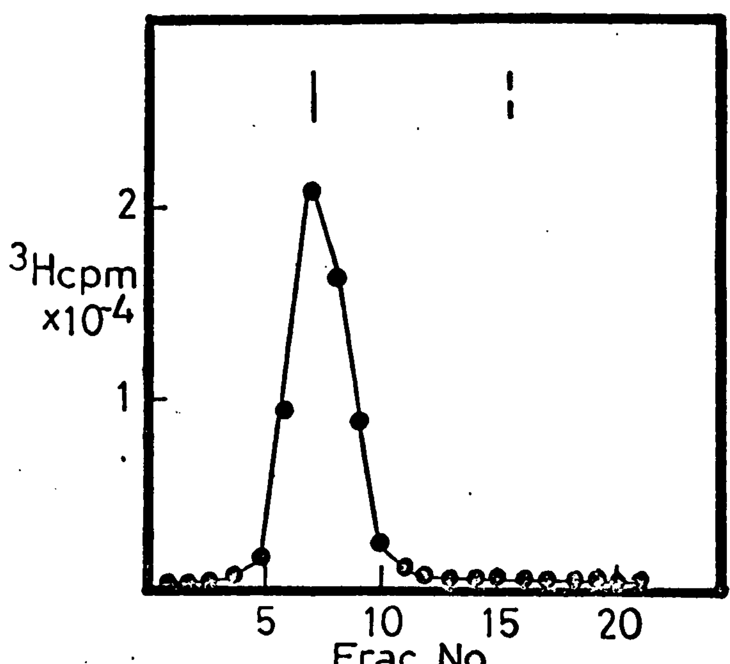

100
80
60
40
20
%P+
1 2 3 4 5 6 7
GENERATIONS

Figure 10. CsCl density gradient DNA profile of a ^{3}H-adenine labeled petite clone isolated from strain 144-2C Gal$^+$ after exposure to 36°C in galactose media. The 4 hour labeling of this petite strain with 20μCi/ml of ^{3}H-adenine in YNBD + X media was carried out in the presence of 200μg/ml of cycloheximide as described in the legend to Figure 3.



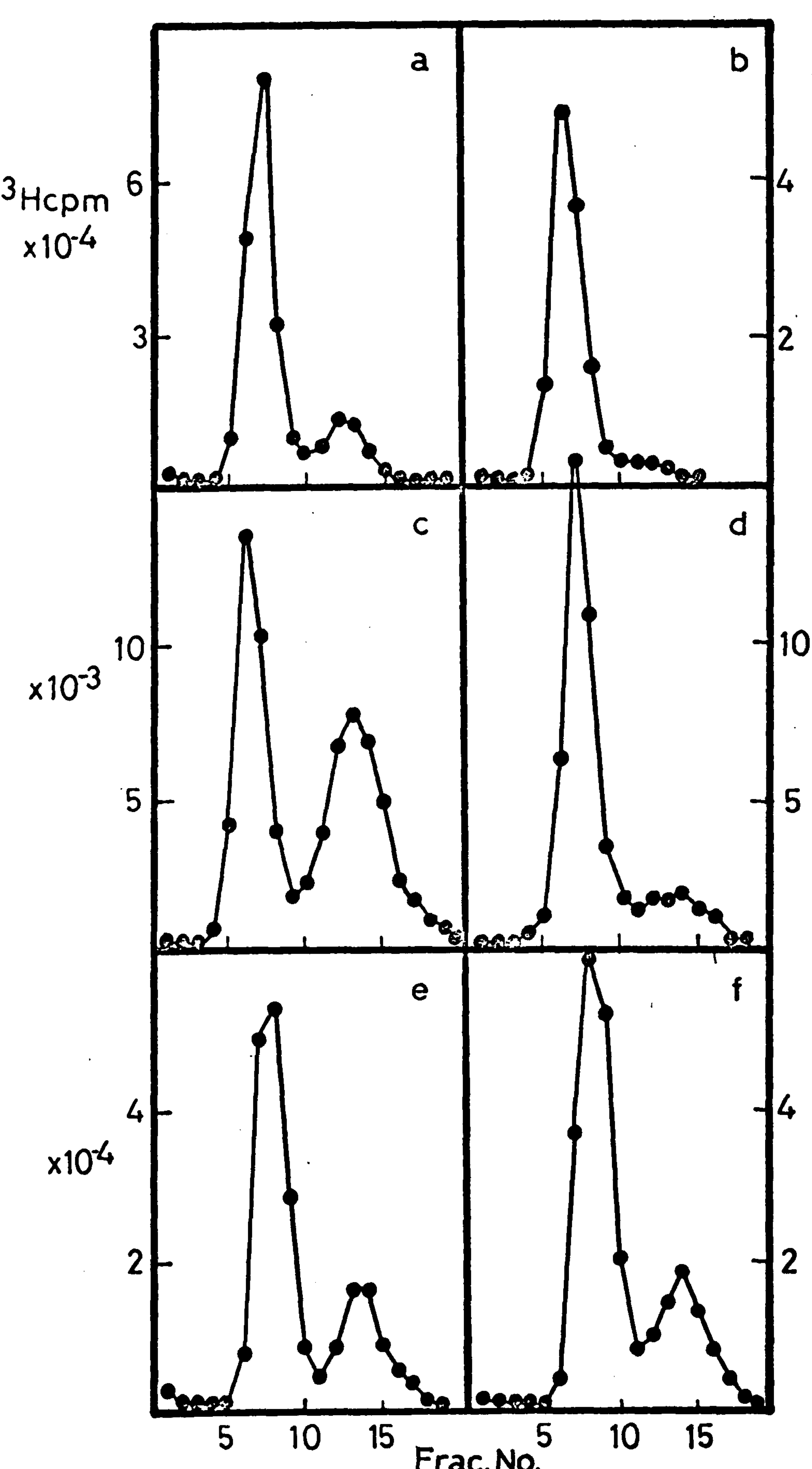
2
1
^{3}Hcpm
x10^{-4}
5
10
15
20
Frac. No.

Figure 11a-f. CsCl density gradient DNA profiles of cells grown in YNBGal + X media in the presence of 20 μCi/ml ^{3}H-adenine for 4 hours.

(a & b) Radioactive incorporation of strain 144-2C Gal^{+} at 24°C (a) and 36°C (b).

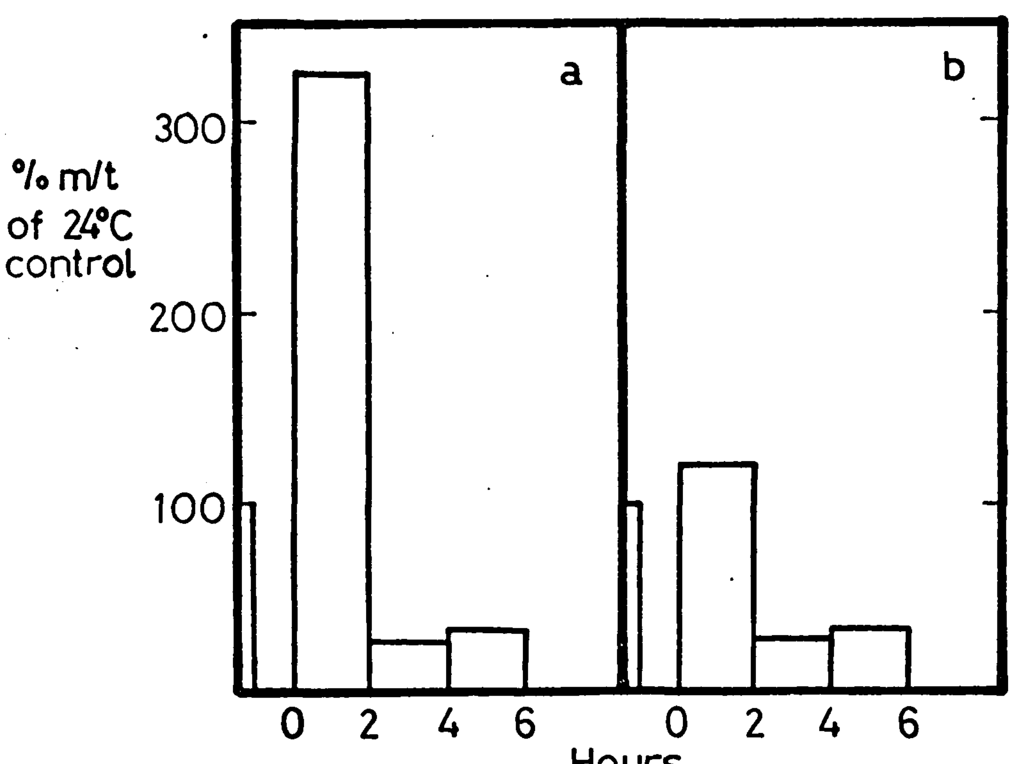
(c & d) Radioactive incorporation of strain 144-2C Gal^{+} in the presence of 200 μg/ml of cycloheximide at 24°C (c) and 36°C (d).

(e & f) Radioactive incorporation of strain 123.1C Gal^{+} at 24°C (e) and 36°C (f).



a
b
c
d
e
f
^{3}Hcpm x10^{-4}
6
3
4
2
x10^{-3}
10
5
10
5
x10^{-4}
4
2
4
2
5
10
15
5
10
15
Frac. No.

Figure 12a-b. Kinetics of mitochondrial DNA synthesis of strain 144-2C at the restrictive temperature in dextrose (a) and glycerol (b) containing medias. Cells grown to mid log phase in either YNBD + X or YNBG + X were divided into equal aliquots which were exposed to 36°C for 0, 2 or 4 hours prior to a two hour labeling with ^{3}H-adenine at 36°C. These cells were then harvested, lysed and their DNA banding profiles analyzed as described in the legend to Figure 3. The mitochondrial/total ratio is plotted for the two hour labeling periods as a percent of the 24°C control (see Materials and Methods).



a
b
300
200
100
% m/t of 24°C control
0
2
4
6
0
2
4
6
Hours

Figure 13. Viable count determinations of strain 144-2C ρ^--3 incubated at 24°C (o—o) and 36°C (●—●) in liquid YEPD media. Cells growing in liquid YEPD media were diluted into 10 ml aliquots of fresh YEPD at a concentration of 5.6×10^3 cells/ml and allowed to grow at either 24°C or 36°C for approximately 32 hours. The number of viable cells in these cultures was monitored during this period by diluting and plating cells from both temperatures onto solid YEPD media, allowing the cells to incubate for 6 days at 24°C, and then counting the number of colonies formed on these plates.

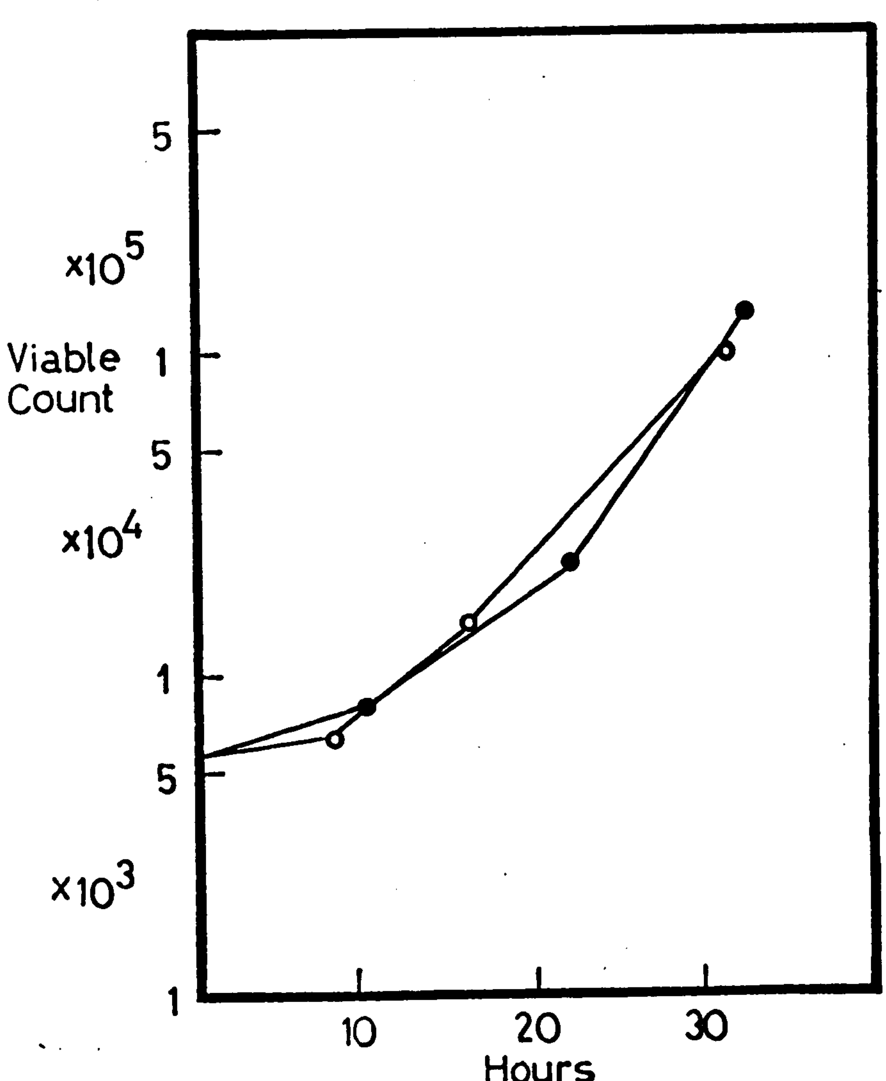

5
x10^5
Viable Count
1
5
x10^4
1
5
x10^3
1
10
20
30
Hours

Figure 14a-b. CsCl density gradient DNA profiles of ^{3}H-adenine labeled petite clones isolated from strain 144-2C ρ^--3 after exposure to 24°C (a) and 36°C (b). The 4 hour labeling of these petite clones in liquid YNBD + X media was carried out using 10 µCi/ ml ^{3}H-adenine in the presence of 200 µg/ml of cycloheximide. The cells were then harvested, lysed and analyzed as described in the legend to Figure 3.

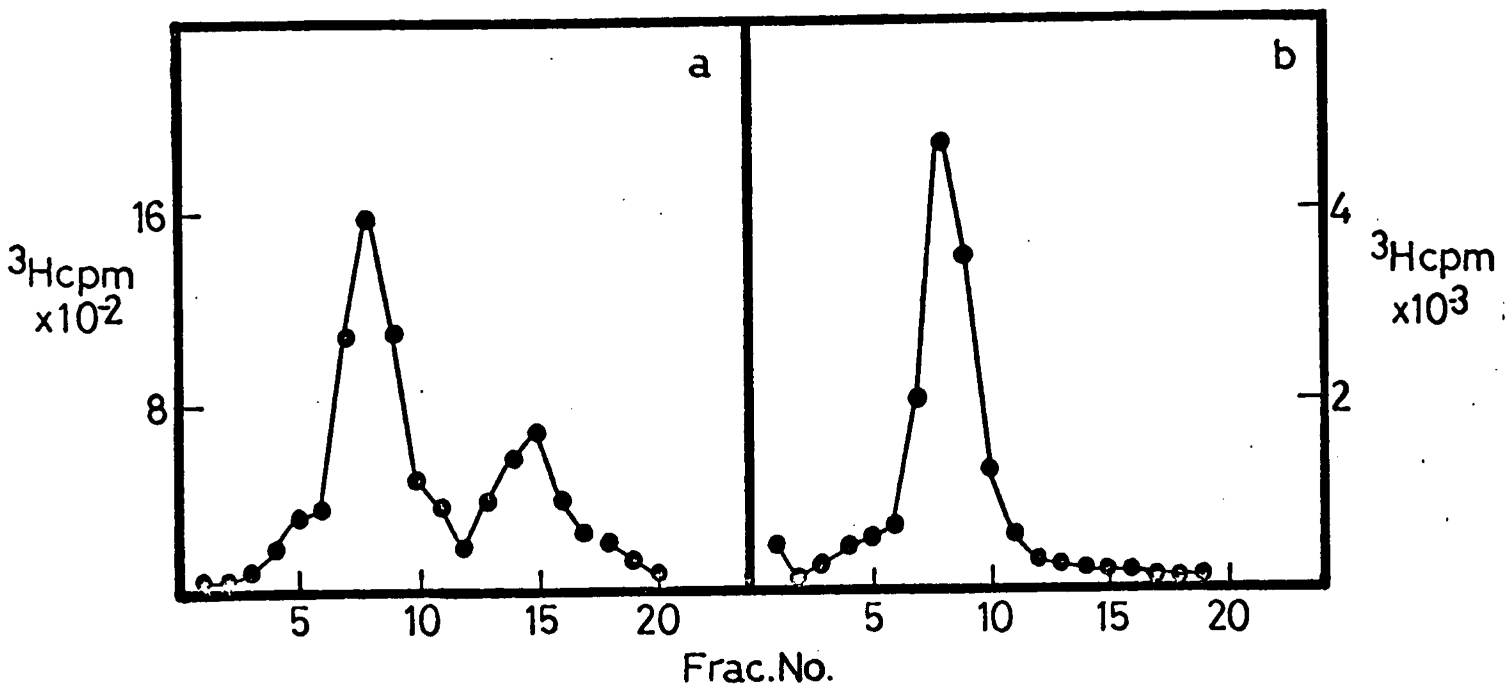

a
b
16
8
3Hcpm
x10-2
4
2
3Hcpm
x103
5
10
15
20
5
10
15
20
Frac.No.

Figure 15. Viable count determinations of strain TS6M4.6 incubated at 24°C o—o and 36°C •—• in liquid YEPD media. Cells growing in liquid YEPD media at 24°C were diluted into two 10 ml aliquots of YEPD at a concentration of 1.3×10^7 cells per ml and allowed to grow at either 24°C or 36°C for 7.5 hours. The number of viable cells in these cultures was monitored as described in the legend to Figure 13.

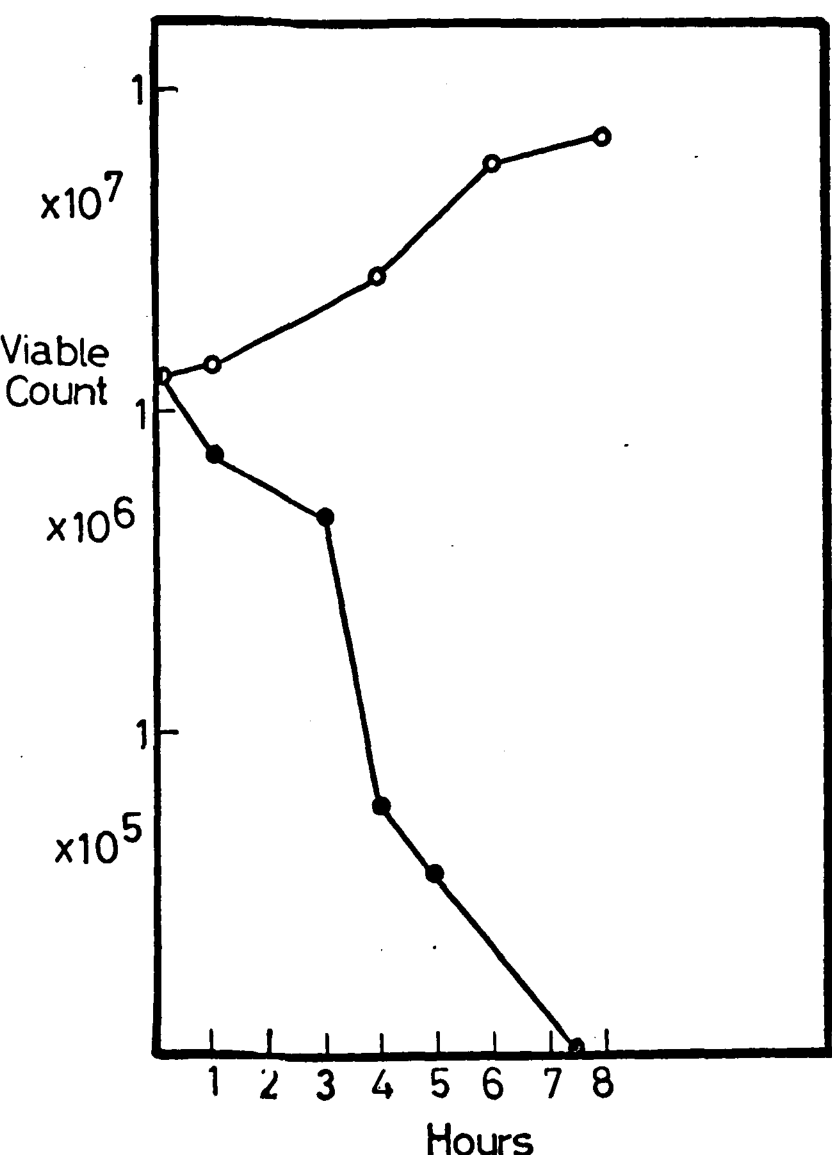

1
x10^7
Viable
Count
1
x10^6
1
x10^5
1 2 3 4 5 6 7 8
Hours

Figure 16. Optical density determinations of strain TS6M4.6 incubated at 24°C o—o and 36°C ●—● in liquid YEPD media. Cells growing in liquid YEPD at 24°C were diluted into two 10 ml aliquots of YEPD at a klett optical density reading of 67, and allowed to grow at either 24°C or 36°C for 8.5 hours. The optical density measurements during this period was determined as described in the Materials and Methods.

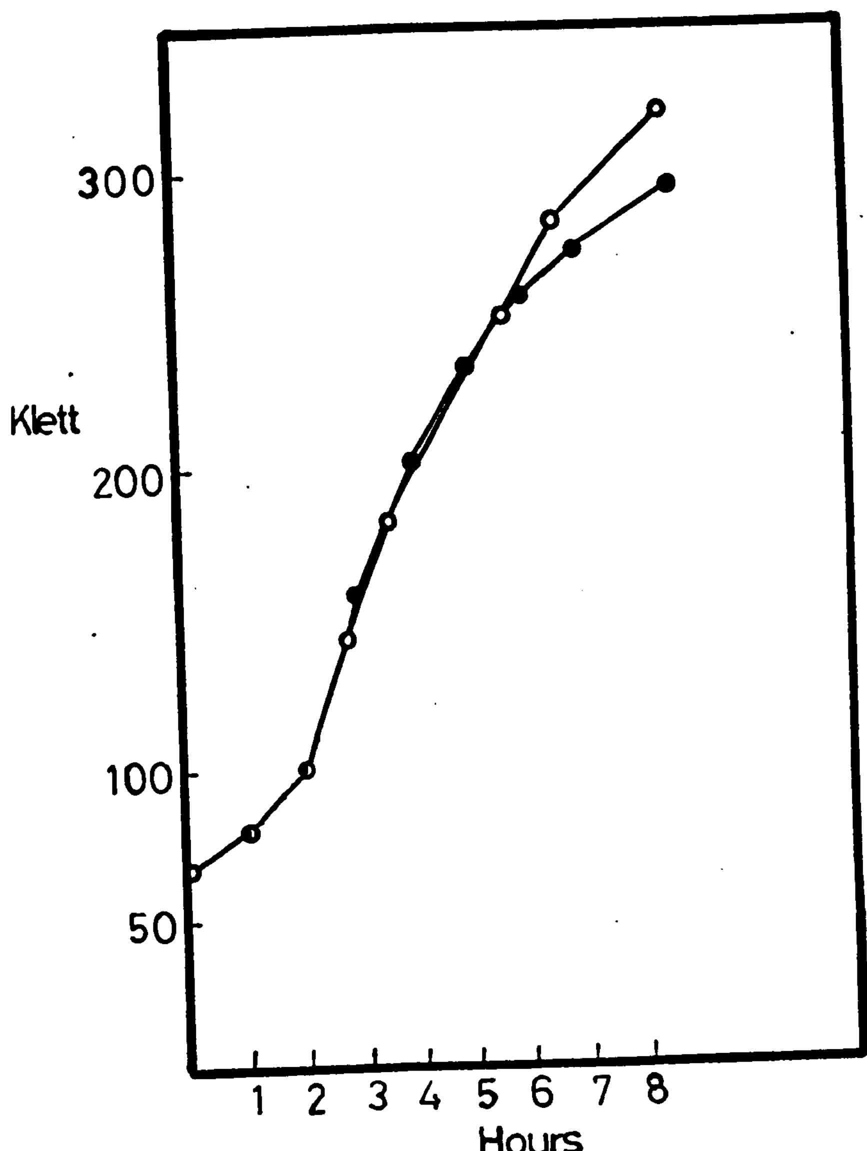

300
Klett
200
100
50
1 2 3 4 5 6 7 8
Hours

Figure 17. Viable count determinations of strain TS6M4.6 incubated at 24°C o—o, 30°C •—• and 36°C •- - -• in liquid YEPD media. Cells growing in liquid YEPD at 24°C were diluted into three 4 ml aliquots of YEPD at a concentration of 2.25×10^7 cells per ml and allowed to grow at either 24°C, 30°C or 36°C for 3 hours. The number of viable cells in these cultures was monitored as described in the legend to Figure 13.

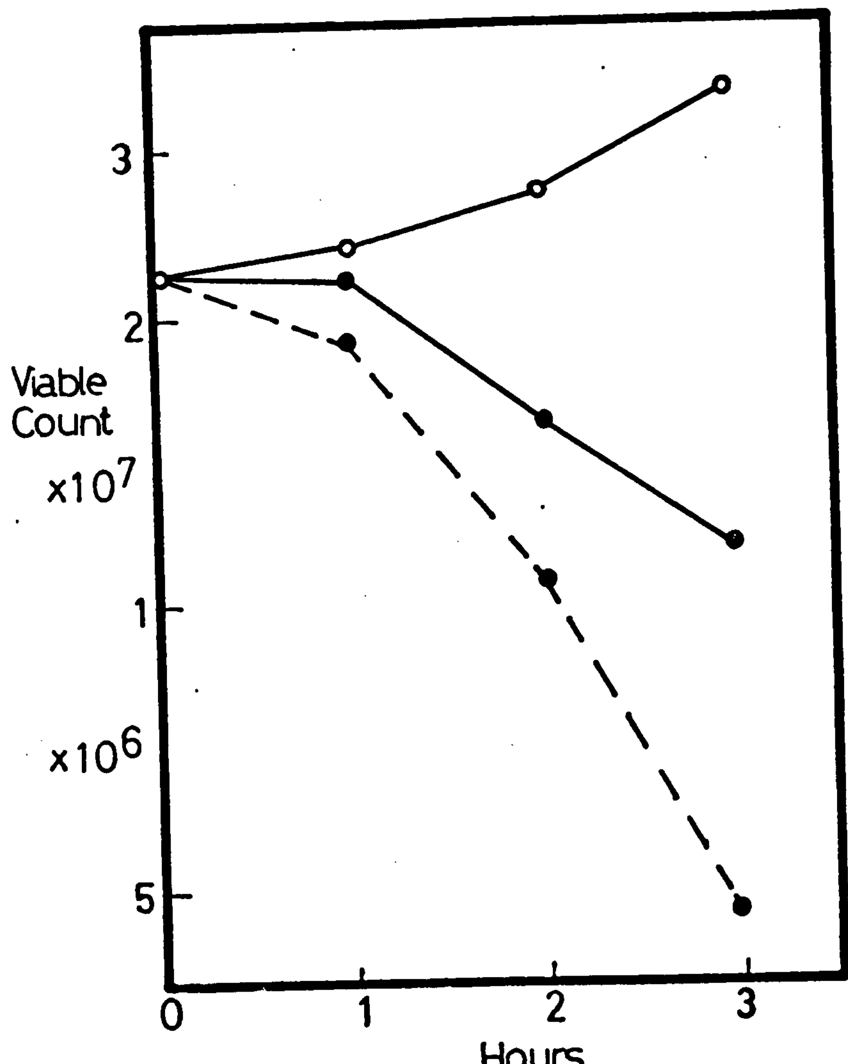

3
2
Viable Count
x10^7
1
x10^6
5
0
1
2
3
Hours

Figure 18. Viable count determinations of strain TS6M4.6 incubated at 36°C ●—● in YNBD, a media unable to support the growth of TS6M4.6. Cells grown in liquid YEPD media were centrifuged, resuspended in YNBD media lacking auxotrophic supplements necessary for the growth of TS6M4.6, and allowed to incubate at 24°C for 6 hours. These starved cells were then incubated for 7 hours at 36°C during which time the number of viable cells in this culture was monitored as described in the legend to Figure 13.

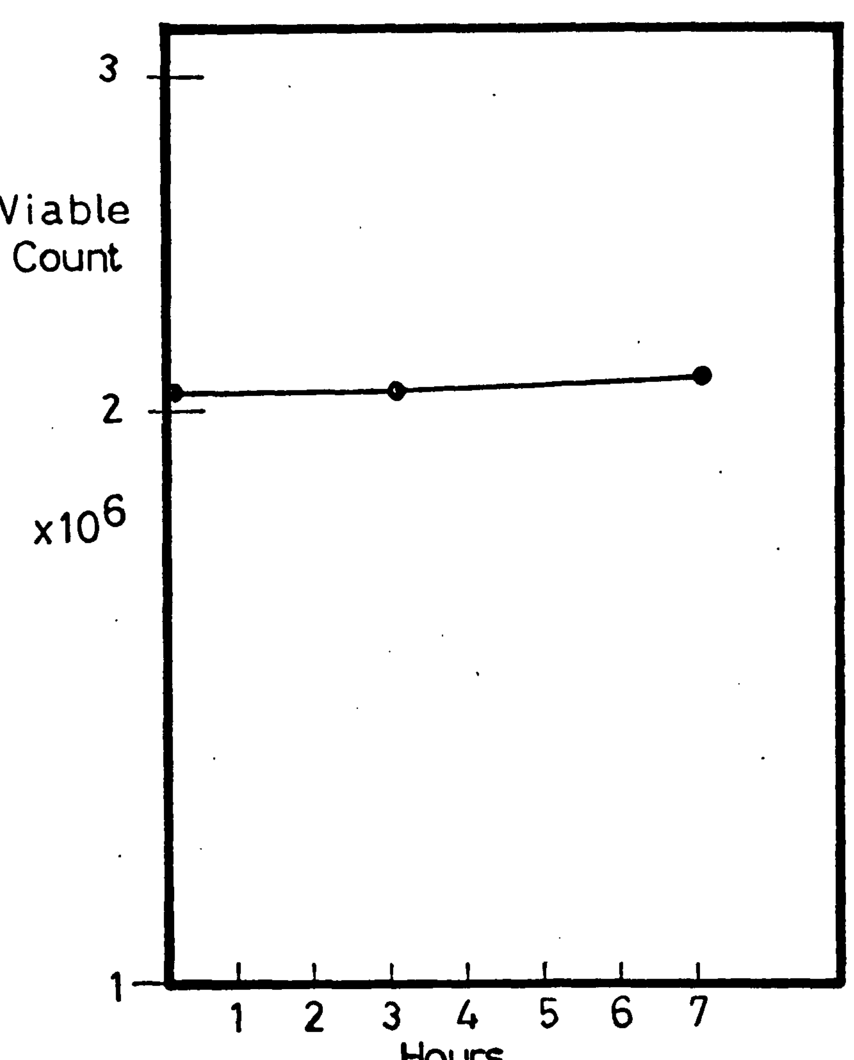

3
Viable
Count
2
x10^6
1
1 2 3 4 5 6 7
Hours

Figure 19. Synthesis of DNA by strain TS6M4.6 at 24°C o—o and 36°C •—• in YNBD + X media. A culture of cells grown to mid log phase in liquid YNBD + X at 24°C was divided into two equal aliquots and then incubated at either 24°C or 36°C in the presence of 9 µg per ml cold adenine and 10 µCi per ml ^{3}H-adenine. At hourly intervals for a 5 hour period, triplicate samples of the radioactively labelled cultures were assayed for the presence of hot alkali stable cold acid precipitable counts, as described in the Materials and Methods. The number of counts per minute presented in this figure represent the average of the triplicate samples.

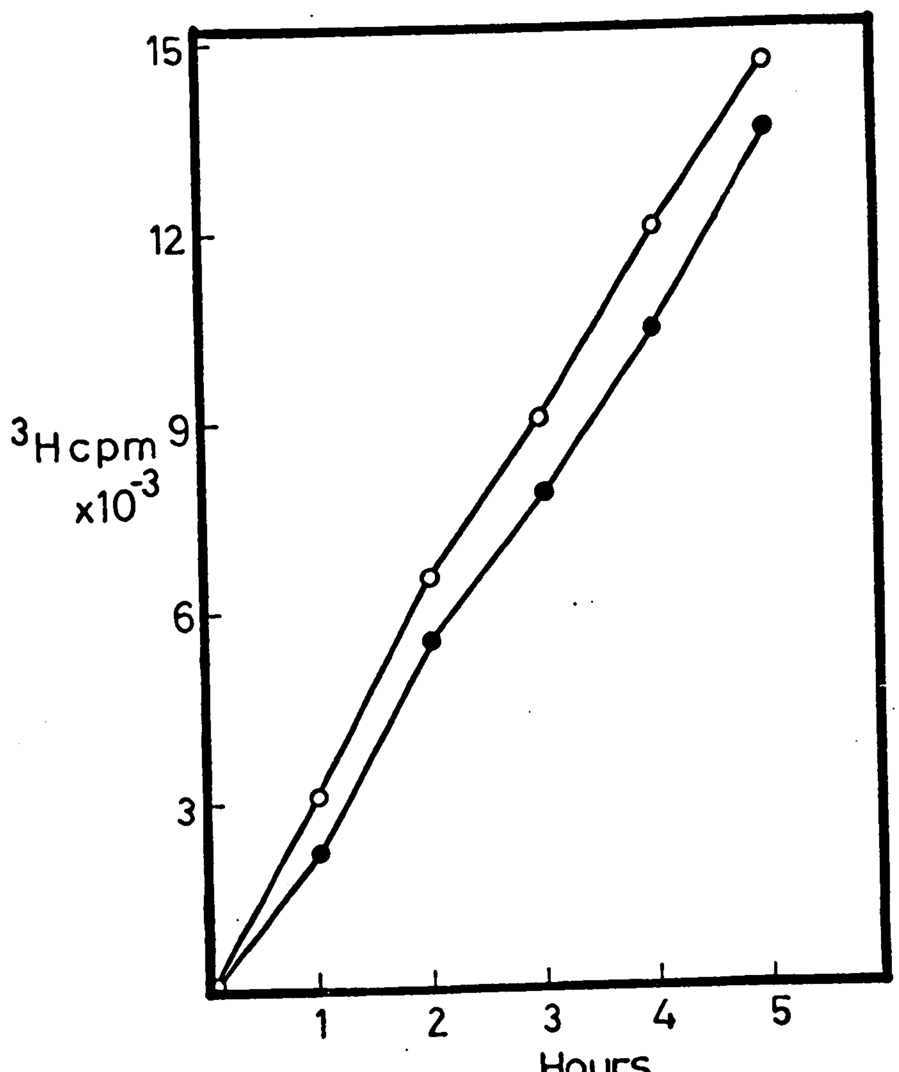

15
12
9
6
3
^{3}H cpm
x10^{-3}
1
2
3
4
5
Hours

Figure 20. Percentage of viable meiotic products produced from diploids initially created in a cross between cells of TS6M4.6 exposed to 36°C for varying lengths of time prior to mating, and strain A364A. Cells of strain TS6M4.6 exposed to 36°C for varying lengths of time (0 - 5 hours) in liquid YEPD, were returned to the permissive temperature and mated with the strain A364A. Resulting diploids were isolated on selective media, allowed to grow for 6 days at 24°C and were then made to undergo meiosis at 24°C on KAc media. The resulting meiotic products were dissected by standard micromanipulation techniques and the spores allowed to grow for 8 days at 24°C. The percentage of viable meiotic products ●—● was determined by the formula: number of viable spores/total number of spores dissected x 100.

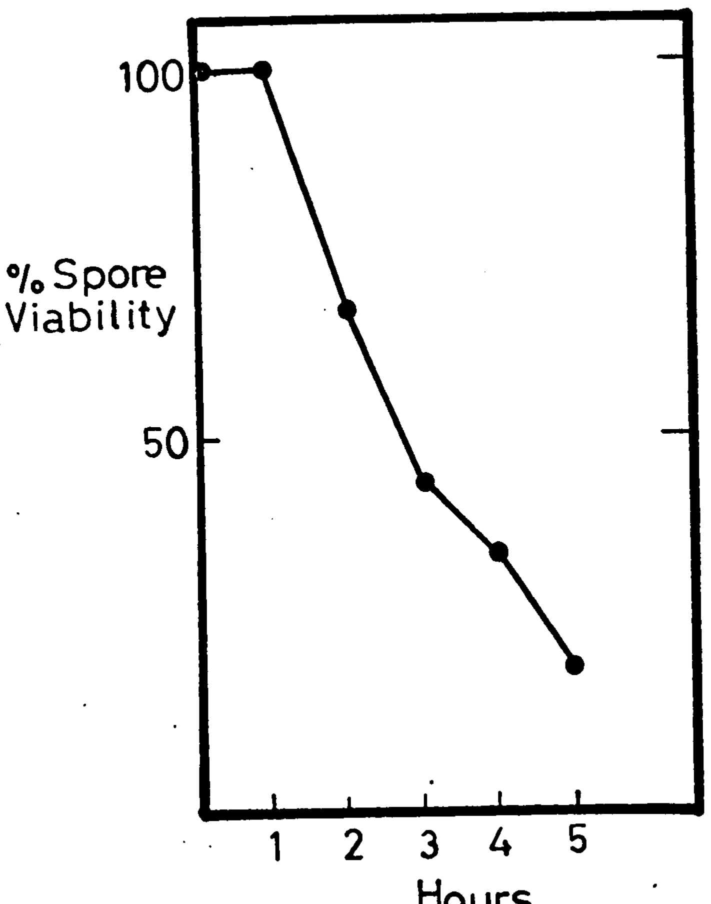
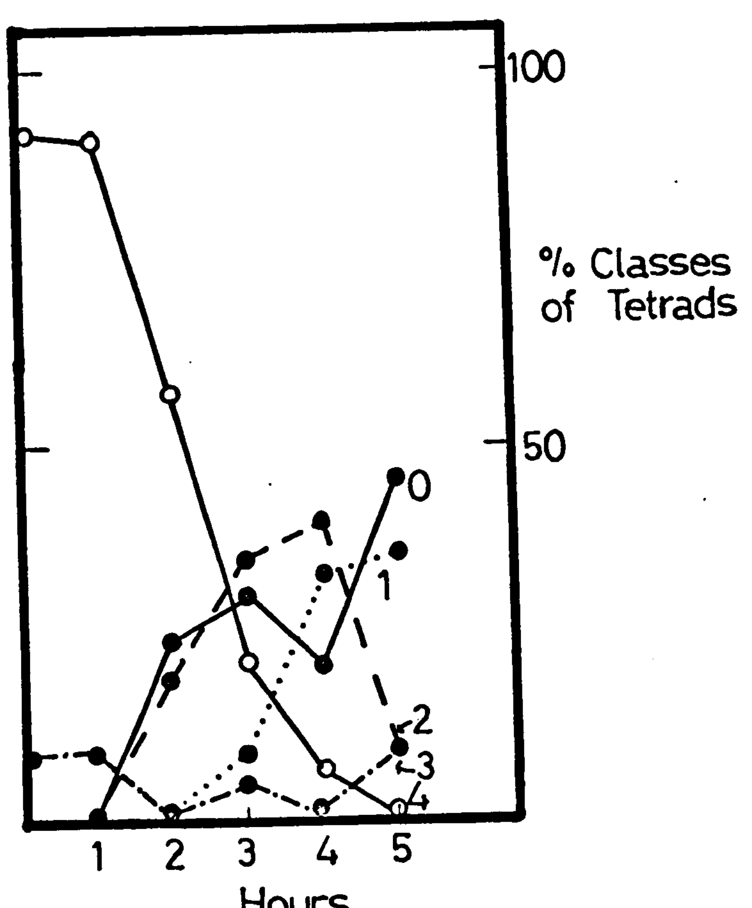
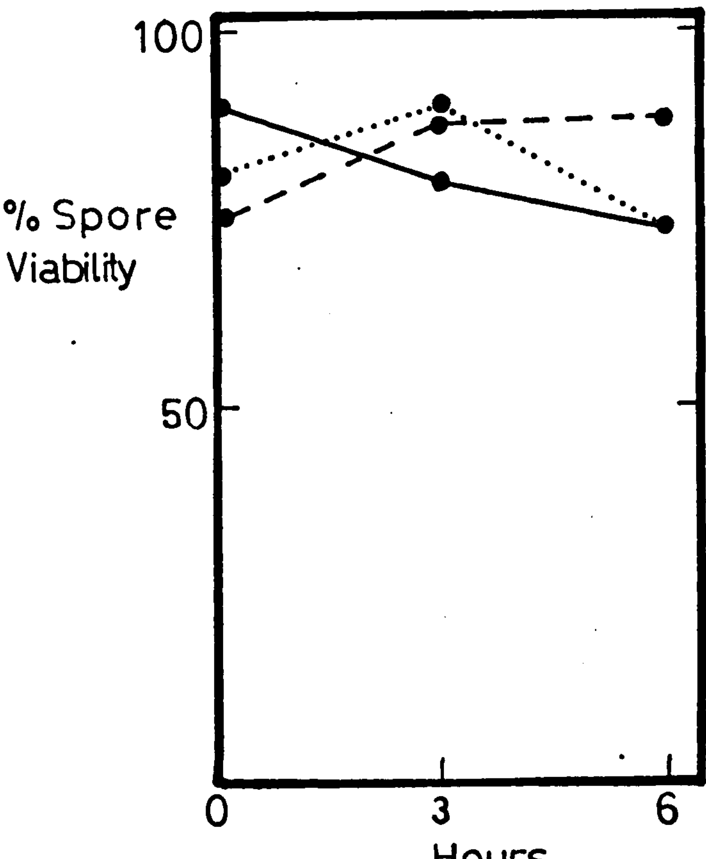

100
50
% Spore
Viability
1
2
3
4
5
Hours

Figure 21. Percentages of the total tetrads yielding that particular number of surviving spores. The diploids described in the legend to Figure 6 yielded asci which produced from 0 to 4 surviving spores. The number of tetrads in these various classes is depicted as a percentage of the total number of tetrads dissected at each time interval. The various classes are depicted as follows: 4 surviving spores o—o, 3 surviving spores ●- · -●, 2 surviving spores ●- - -●, 1 surviving spore ●·· ··●, and 0 surviving spores ●—●.



100
50
% Classes
of Tetrads
0
1
2
3
4
1
2
3
4
5
Hours

Figure 22. Percentage of viable meiotic products produced from diploids initially created in a cross between cells of several cdc mutants, exposed to 36°C for varying lengths of time prior to mating, and strain 123.1C. Cells of strains cdc4 ●—●, cdc 7 ●····● and cdc 8 ●- - -● exposed to 36°C for varying lengths of time (0 to 6 hours) in liquid YEPD, were returned to the permissive temperature and mated with the strain 123.1C. The percentages of viable meiotic products produced by these diploids was determined as described in the legend to Figure 20.


100
50
% Spore
Viability
0
3
6
Hours

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