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Bromodeoxyuridine 5'-Monophosphate Incorporation into Yeast Nuclear and Mitochondrial Deoxyribonucleic Acid

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Standard laboratory yeast strains can be enriched for thymidine 5'-monophosphate (TMP) uptake derivatives that generate only a low percentage of respiratory-deficient colonies (petites) under inhibition of TMP biosynthesis. Such mutants incorporated bromodeoxyuridine 5'-monophosphate (BrdUMP) into both nuclear and mitochondrial deoxyribonucleic acid (mtDNA); however, they showed a selectivity for TMP over BrdUMP incorporation. The preferential incorporation of [^{3}H]TMP or BrdUMP into mtDNA was strain dependent. The density increments after growth in the presence of BrdUMP reached 50 mg/ml for nuclear DNA and 22 mg/ml for mtDNA in CsCl gradients. Density shifts corresponding to 4% bromouracil substitution were easily detected. Preliminary density transfer experiments confirm that mtDNA does not replicate in synchrony with nuclear DNA.

The study of deoxyribonucleic acid (DNA) synthesis in most organisms is facilitated by specific labeling with radioactive thymine or thymidine. The thymidine analogue, 5-bromodeoxyuridine, has also been used extensively to follow DNA replication in density transfer experiments by analyzing the buoyant density distribution in CsCl gradients. In addition, 5-bromodeoxyuridine labeling, when used in combination with a radioactive DNA precursor, has contributed to distinguish further between DNA replication and repair (10, 17). Unfortunately, the yeast *Saccharomyces cerevisiae* lacks thymidine kinase (9) and therefore does not incorporate thymine, thymidine, or 5-bromodeoxyuridine. Consequently, yeast DNA has been labeled with other radioactive bases or nucleotides with the attendant problem of incorporation of label into ribonucleic acid. To overcome this problem, mutants have been isolated (1, 2, 5, 6, 15, 20) after selection for growth when the de novo pathway for deoxythymidine 5'-monophosphate synthesis was blocked through the combined action of aminopterin, an inhibitor of dihydrofolate reductase, and sulfanilamide, an inhibitor of folate synthesis (1, 15, 20). The use of these mutants has some drawbacks. First, high levels of exogenous thymidine 5'-monophosphate (TMP) are necessary for optimal incorporation, presumably, because a phosphatase degrades TMP to thymidine and inorganic phosphate (5). Secondly, perhaps as a consequence of the selection procedure (2), the mutants produce a high percentage of petite colonies, which render them impractical for experiments dealing with wild-type mitochondrial DNA (mtDNA). For these, or perhaps for other reasons, such mutants have as yet not been used in studies of DNA synthesis.

Because of the limitations of the present procedures, it was desirable to find conditions under which yeast strains would not produce petites during TMP uptake and which would incorporate deoxybromouridylate (BrdUMP) quantitatively. The increase in buoyant density of DNA from yeast grown in the presence of BrdUMP would be an unequivocal way of ascertaining the specific incorporation of the exogenous nucleotide into replicating DNA. In this report, we describe the isolation of stable derivatives with low requirements for TMP from a number of standard laboratory strains, using a modification of existing methods. Furthermore, we describe the incorporation of BrdUMP into the nuclear and mtDNA of these strains.

MATERIALS AND METHODS

Media. Complete medium (YPD) contained 1% yeast extract, 2% peptone, and 2% dextrose (2% agar was added for plates). Glycerol-ethanol (YPEG) medium contained 2% glycerol and 3% ethanol instead of glucose. Dextrose-glycerol medium contained 0.1% dextrose and 3% glycerol as carbon sources and was used to differentiate between grande and petite colonies.

Labeling medium (YPD-2) was that of Brendel and Haynes (2), as modified by Wickner (20). It consisted of 0.25% yeast extract, 1% peptone, and 2% dextrose. To this medium, after autoclaving, were added filter-sterilized solutions of sulfanilamide (Sigma Chemical Co.), 6 mg/ml, aminopterin (K &

K Laboratories), 50 μg/ml, and deoxy-TMP (Sigma Chemical Co.) as needed. The pH of the medium was adjusted to be between 5.6 and 5.8. BrdUMP was synthesized by using the method of Michelson et al. (18). The yeast α factor was isolated according to the method of Bucking-Throm et al. (3).

Yeast strains. A364A, T108 *tupl* (a *ade1, ade2, his7, gal1, ura1, lys2, trp1*) (13) was obtained from Reed Wickner. D273-10B (α, wild type) was obtained from F. Sherman. K8 (α, *leu1, trp5, ura1*) was obtained from E. Jones. The cell division cycle mutants *cdc21, cdc4,* and *cdc23* were obtained from the Yeast Genetic Stock Center at Berkeley.

Radioactive compounds. [^{3}H]TMP (40.8 Ci/mmol), [8-^{14}C]adenine (57 mCi/mmol), and [2-^{3}H]-adenine (20.2 Ci/mmol) were obtained from New England Nuclear Corp.

DNA isolation. The methods used for spheroplast formation, preparative CsCl density gradient centrifugation, and analytical density gradient centrifugation were exactly as described by Goldthwaite et al. (8).

Enrichment for TMP uptake mutants. All strains, including T108 *tupl*, were inoculated with a low inoculum in YPD-2 medium supplemented with aminopterin and sulfanilamide (SAT medium) and 25 μg of TMP per ml. Growth was monitored with a Klett-Summerson photometer equipped with a red filter. Enrichment in TMP uptake derivatives was obtained by successive transfers in SAT medium after the culture reached stationary phase. Three to five such transfers were often sufficient. To select the population of yeast cells that were capable of growing in the presence of aminopterin and sulfanilamide without becoming petite, cells were also grown on YPEG plates as an inoculum for the next round of growth in SAT and TMP. When a culture had achieved optimal growth on SAT and TMP medium (3×10^7 cells/ml or above at stationary phase), single-colony derivatives that showed minimal growth in the absence of TMP and maximal growth in the presence of no more than 25 μg of TMP per ml were picked.

Labeling with BrdUMP. Yeast cells were grown in SAT with the addition of varying amounts of TMP and BrdUMP. The culture flasks were wrapped in foil and handled in the absence of fluorescent light. Labeled DNA was isolated with the same precautions.

RESULTS

Growth in the presence of TMP. Following the procedure just described, TMP uptake derivatives were isolated from the six strains used. It is probable that such derivatives could be obtained from most other, if not all, laboratory yeast strains. The number of transfers required to select nonpetite-producing TMP uptake derivatives was strain dependent. Some strains, such as D273-10B, were able to grow initially in the presence of SAT plus TMP to some extent even without enrichment. As previously reported, thymine and thymidine could not substitute for TMP. The critical step for optimal TMP uptake is to obtain conditions under which there is minimal growth in the absence of TMP. The inhibition of de novo synthesis of TMP through the combined action of aminopterin and sulfanilamide is very sensitive to the influence of parameters such as the pH of the medium, the age of the culture, and the size of the inoculum. In the absence of TMP, a culture is capable of undergoing three and one-half doublings, possibly because of the presence of a pre-existing pool. Consequently, to ensure optimal uptake of exogenous TMP by a cell culture, it is necessary to start the culture with a low inoculum, i.e., not more than 10^6 cells/ml. Furthermore, the energy source may also be important since, with strain T108V, the de novo synthesis was not blocked when the glucose in the medium was replaced by ethanol and glycerol.

Figure 1 shows a comparison of the growth curves of the cell division cycle mutant *cdc21* before and after enrichment in TMP uptake mutants. The growth curves shown in Fig. 2 were obtained with Wickner's mutant, T108 *tupl*, which, after having undergone further selection with our procedure, was designated as

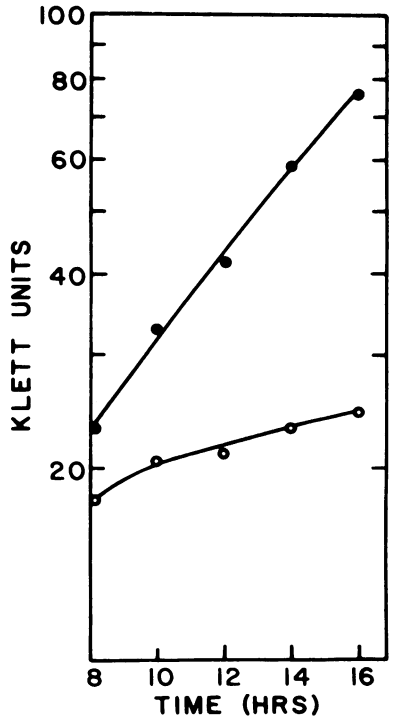

FIG. 1. *Growth of cdc21 in SAT plus TMP (25 μg/ml) before (○) and after (●) enrichment of culture in a TMP uptake mutant.*

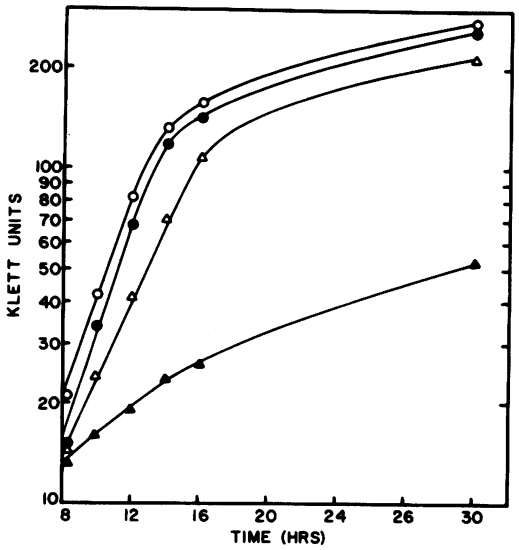

FIG. 2. *Effect of TMP concentration on growth of T108V. The cells were grown at 30 C in SAT: ▲, without TMP; △, TMP, 5 μg/ml; ●, TMP, 10 μg/ml; ○, TMP, 25 μg/ml.*

T108V. One can see that the growth rate, in the presence of drugs, responds to as little as 3 μg of TMP per ml in the medium and increases until the TMP concentration in the medium reaches 10 μg/ml. Further increases in TMP concentration do not increase the rate of growth. At a concentration of 10 μg/ml in TMP, the composition of the YPD-2 medium probably becomes growth limiting because of its low content of peptone and/or yeast extract. The comparison of single-colony isolates from a culture of *cdc21* enriched for TMP uptake mutants showed that the culture included several mutants with quite different growth characteristics in the presence of TMP. In the cultures derived from the single colonies examined, the maximum amount of growth reached by a culture in SAT and TMP (25 μg/ml) medium seems to be a stable genetic characteristic. Raising the concentration of exogenous TMP did not result in increased growth. Crosses with a wild-type strain result in diploids that connot grow an SAT-TMP medium; thus, as reported by others (1, 2, 21), the TMP uptake trait is recessive.

Uptake of [^{3}H]TMP. To determine the relative incorporation of TMP into nuclear and mtDNA, culture of T108V were grown to early logarithmic phase in SAT and TMP (25 μg/ml) with either 10 μCi of [^{3}H]TMP or 2.5 μCi of [^{14}C]adenine per ml. The cultures were harvested, and aliquots of each were mixed to compare the relative counts. The cells were converted to spheroplasts, lysed, and centrifuged in a preparative CsCl gradient. The results (Fig. 3) show that the ratio of labeled nuclear DNA to mitochondrial DNA is the same in cells labeled with [^{3}H]TMP and in cells labeled with [^{14}C]adenine; consequently, in T108V there is no preferential labeling of either DNA species.

Incorporation of BrdUMP. Cultures of T108V in SAT plus 25 μg of TMP per ml were grown for 24 and 48 h in the presence of different concentrations of BrdUMP. Total cell DNA was isolated and analyzed by analytical CsCl density gradient centrifugation. Both the newly synthesized nuclear DNA and mtDNA showed increases in buoyant density. To distinguish which peaks represented nuclear DNA and which represented mtDNA without isolating them separately, the DNA of an ethidium bromide (EB)-induced ρo derivative of T108V lacking mtDNA was isolated and banded in a CsCl density gradient. Several such analyses showed that the T108 ρo petite had no detectable mtDNA. The nuclear DNA band was used to identify the corresponding DNA species in T108V.

Values for the buoyant density of DNA from cultures grown with increasing BrdUMP con-

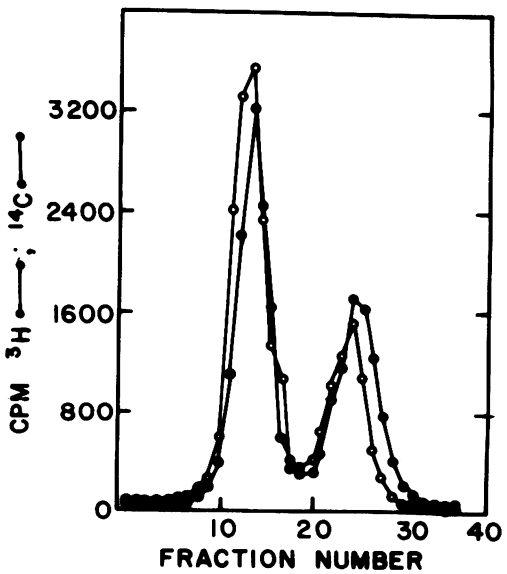

FIG. 3. *Density gradient centrifugation of two cultures of T108V. Culture A was grown to logarithmic phase in the presence of 10 μCi of [^{3}H]TMP per ml; culture B was grown in the presence of 2.5 μCi of [^{14}C]adenine per ml. Aliquots of the two cultures were mixed. The lysates were centrifuged in a preparative CsCl gradient. Symbols: ○, [^{3}H]TMP; ●, [^{14}C]adenine.*

centrations are reported in Table 1. For given concentrations of TMP and BrdUMP the buoyant density values were usually reproducible within 1 mg/ml when T108V and T108V ρo EB were grown under exactly the same conditions.

It can be seen in Fig. 4 that the increase in buoyant density of both species of DNA is directly proportional to the concentration of BrdUMP in the medium, when the concentration of TMP is constant. No data are presented for mtDNA for concentrations of BrdUMP higher than 25 μg/ml, since mtDNA is presumably degraded under these labeling conditions. The slope of the buoyant density increase of mtDNA as a function of BrdUMP concentration is steeper than that of nuclear DNA. This may reflect either a preferential incorporation of BrdUMP into mtDNA or the higher percentage of thymine residues in mtDNA. The percentage of bromouracil (BU) substitution for thymine was calculated according to the method of Flory and Vinograd (7). In Fig. 5, the buoyant densities of both DNAs are expressed as percentage of BU substitution in one strand. The increase in percentage of BU substitution as function of BrdUMP concentration is the same for both DNAs. Accordingly, one can conclude that in T108V there is no preferential incorporation of BrdUMP by either nuclear or mtDNA. To see if yeast, like *Escherichia coli*, shows a marked selectivity for thymine over BU at the nucleotide level (11), the increase in buoyant density was expressed as a function of the percentage of BrdUMP in the total (BrdUMP + TMP). The incorporation of BrdUMP into DNA is inversely related to the relative concentration of TMP in the medium (Fig. 6). Consequently, yeast, like *E. coli*, also shows a selectivity for TMP over BrdUMP.

TABLE 1. *Buoyant density increase and BU substitution in yeast DNA as a function of BrdUMP concentration in the medium*[a]

BrdUMP (μg/ml)	Nuclear DNA		mtDNA	
	Buoyant density (g/ml)	BU substitution (%)	Buoyant density (g/ml)	BU substitution (%)
0	1.698	0	1.681	0
3	1.701	4.6	1.684	3.7
5	1.705	10.6	1.688	8.7
10	1.707	13.6	1.688	8.7
15	1.711	19.7	1.697	19.4
25	1.717	28.8	1.703	26.9
50	1.728	45.5		
75	1.736	57.6		
85	1.748	71.2		

[a] TMP concentration was 24 μg/ml in SAT medium in all the experiments.

Incorporation of BrdUMP into mtDNA in the absence of nuclear DNA replication. BrdUMP incorporation into mtDNA was followed in a culture grown in the presence of α factor, which inhibits nuclear DNA replication without inhibiting mtDNA synthesis (19). A culture of T108V was grown to logarithmic phase and combined with the control culture, yeast α factor (0.2 ml) was then added to the culture, and, after 1 h, [^{3}H]adenine and BrdUMP (15 μg/ml) were added. The culture was harvested after 8 h, converted to spheroplasts, lysed, and centrifuged in a preparative CsCl

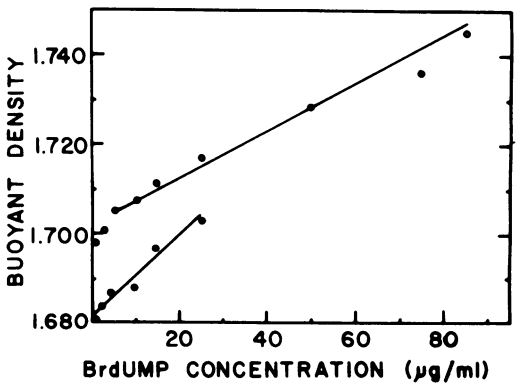

FIG. 4. *Buoyant density increases of nuclear DNA and mtDNA as a function of BrdUMP concentration. Cultures of T108V were grown in SAT plus 24 μg of TMP per ml and different concentrations of BrdUMP. Total cell DNA was isolated and analyzed by analytical CsCl density gradient. Symbols: ○, nuclear DNA; ●, mtDNA.*

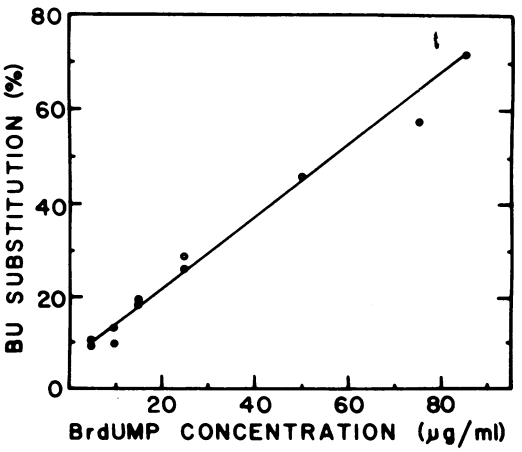

FIG. 5. *Percentage of BU substitution (expressed as single-strand incorporation) in nuclear DNA and mtDNA as a function of BrdUMP concentration. Symbols: ○, nuclear NDA; ●, mtDNA.*

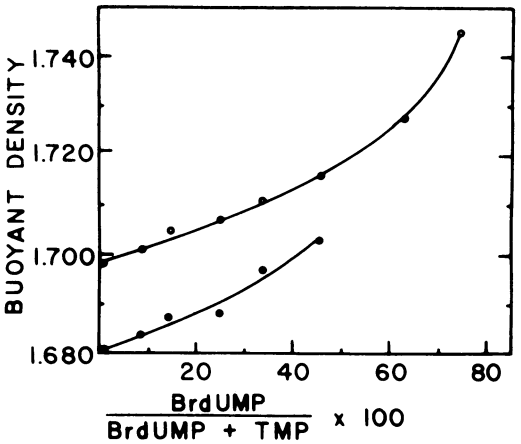

FIG. 6. *Percentage of BU substitution expressed in buoyant density as a function of the percentage of BrdUMP/(BrdUMP + TMP) in the growth medium. Symbols: ○, nuclear DNA; ●, mtDNA.*

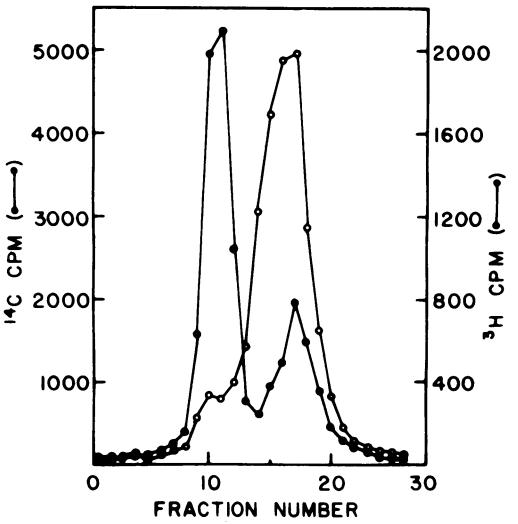

FIG. 7. *MtDNA labeling with BrdUMP in the absence of nuclear DNA replication. A culture was grown to early logarithmic phase in SAT plus 25 μg of TMP per ml and then α factor (0.2 ml) was added to the culture, and, after 1 h, 15 μg of BrdUMP and 10 μCi of [^{3}H]TMP per ml were added. The culture was harvested 8 h later, and an aliquot was combined with an aliquot of a control culture grown in the presence of 2.5 μCi of [^{14}C]adenine per ml. Lysates were prepared and centrifuged in a preparative CsCl gradient. Symbols: ●, culture without α factor; ○, culture with α factor and BrdUMP.*

gradient. In a similar experiment with added α factor, samples were also taken for analytical ultracentrifugation in CsCl. Figures 7 and 8 show that, in the presence of α factor there is no BrdUMP incorporation into nuclear DNA, whereas the buoyant density of mtDNA increases to the same value observed in the absence of α factor. The results of a preliminary study of the kinetics of BrdUMP uptake are presented in Fig. 9. Although the buoyant density shifts are small because of the low concentrations of BrdUMP used (5 μg/ml) in this experiment, a number of conclusions can be drawn regarding the replication pattern of both DNAs. With nuclear DNA, the buoyant density shifts resulting from BrdUMP incorporation reflect those expected from its semiconservative replication (16). At the first doubling, there is one band at a hybrid density, at the second doubling, there are one "hybrid" band and one "heavy" band, and, at the third doubling and thereafter, there is one band at the heavy density. With mtDNA the maximum buoyant density for a given BrdUMP-to-TMP ratio is obtained at one doubling of the culture and does not increase with incubation time.

The above experiment, and another done in the presence of α factor, indicate that the pattern of mtDNA replication is quite different from that of nuclear DNA. At the first doubling, mtDNA shows a shoulder at a heavier density, whereas the bulk of this DNA species still bands at the "light" density. After a second doubling, most of the DNA is at the heavier density and a small fraction is still at the light density. This pattern does not change even after 24 h of growth in the presence of BrdUMP. However, after 24 h of growth at concentrations higher than 15 μg/ml, all of the mtDNA is shifted to the heavier density; there is no unreplicated, light DNA remaining. At all times, mtDNA does not show any intermediate density between the light density and the heavy density. However, due to the small density shifts in this experiment, a small amount of DNA of hybrid density might not have been resolved. Further experiments are in progress to determine the distribution of the label in the mtDNA molecule. The problem is complicated by the possibility of extensive recombination between the mtDNA molecules.

BrdUMP uptake by other strains. The incorporation of BrdUMP into DNA was studied in other strains besides T108V. D273-10B was chosen because it is a wild-type strain and *cdc21* because it is widely used in studies of nuclear and mtDNA replication.

DNA was isolated from cultures of D273-10B grown in the presence of SAT plus 50 μg of BrdUMP per ml. One culture was derived from D273-10B without enrichment for TMP uptake

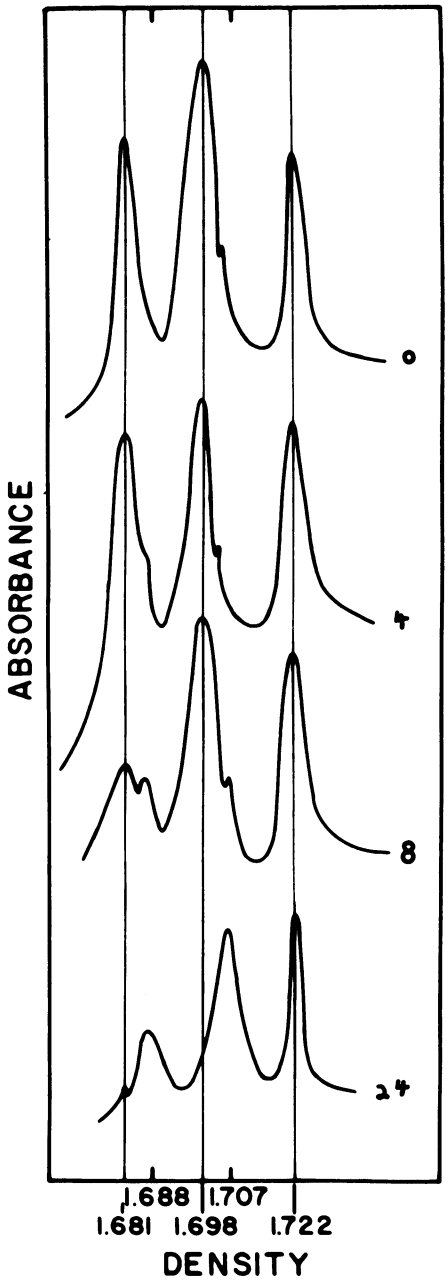

FIG. 8. *Buoyant density profiles of the DNA of a culture grown in SAT, 25 μg of TMP per ml, and 5 μg of BrdUMP per ml in the presence of α factor for 4 and 8 h. Hour 0 is before the addition of α factor and BrdUMP, and h 24 is the control culture grown in the presence of BrdUMP for 24 h without α factor. The reference is Bacillus subtilis phage (PBS2) DNA, whose density is taken to be 1.722 g/ml.*

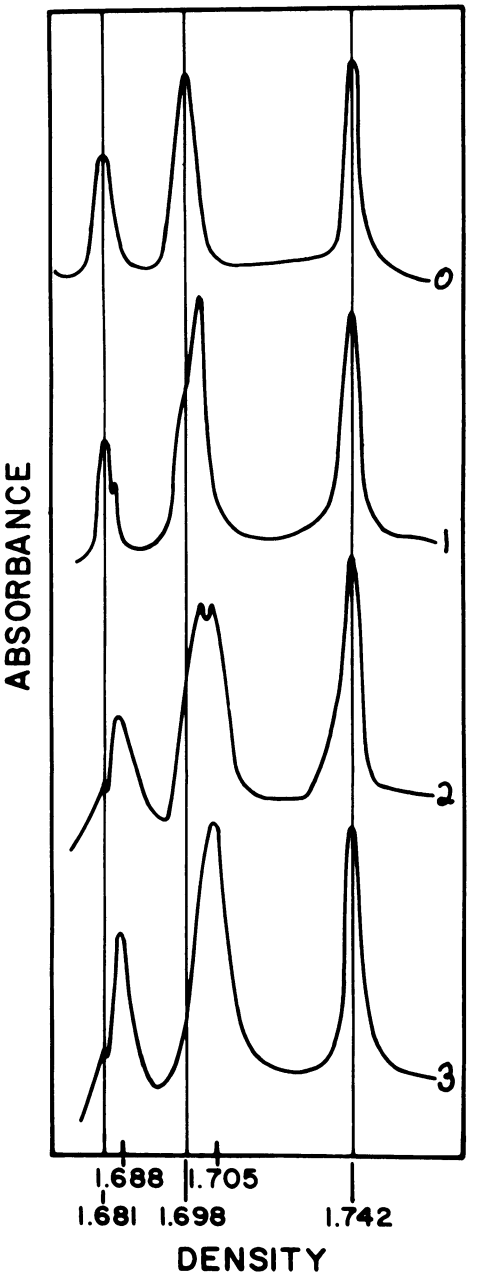

FIG. 9. *Analytical CsCl gradients of whole-cell DNA after the transfer of a culture to medium containing 5 μg of BrdUMP per ml. 0–3, Number of doublings of the culture. The reference is B. subtilis phage (SP8) DNA (density, 1.742 g/ml).*

mutants, and the other was derived from D273-10B after enrichment for growth in SAT plus TMP, as described in Materials and Methods. Both cultures incorporated BrdUMP into DNA.

In the culture not subjected to mutant enrichment, there was a 21% BU substitution for nuclear DNA and a 20% substitution for mtDNA. In the strain enriched for TMP uptake mutants, there was a 38% substitution for nuclear DNA; however, as was the case in T108V grown in the same BrdUMP concentration, the BU-labeled mtDNA was degraded. Strain *cdc21*, after enrichment for TMP uptake mutants, was also tested for BrdUMP incorporation. In the presence of 25 μg of BrdUMP per ml, the BU substitution was 25% for the nuclear DNA and 31% for the mtDNA. In this case there is a preferential labeling of mtDNA compared to nuclear DNA. In the presence of SAT plus TMP, T108V generates from 5 to 10% petites. When grown in 5 μg or more of BrdUMP per ml, the percentage of petites can vary between 20 and 90%. Even at the highest concentration in BrdUMP there are always at least 10% grande colonies. When these grande colonies were tested for resistance to petite induction by BrdUMP, it was found that, again, 10% of the population was composed of grande colonies. This could be analogous to Hanawalt's observation (12) that in *E. coli* some of the parental DNA never incorporates BU. The induction of petites by BrdUMP increases progressively during the growth cycle and reaches its maximum at the end of the logarithmic phase. BrdUMP does not affect viability of yeast at concentrations below 10 μg/ml. At higher concentrations there is a progressive growth inhibition and cell death. However, even at concentrations of 85 μg/ml the rate of BU incorporation in nuclear DNA is the same as at the lower concentrations. Since yeast lacks thymidine kinase (9), one may expect that a culture of yeast grown in the presence of the thymidine analogue of bromodeoxyuridine would not incorporate BU into DNA. This was demonstrated when DNA isolated from such a culture was banded in a CsCl gradient: neither nuclear nor mtDNA showed any increase in buoyant density.

DISCUSSION

The main objective in this work was to devise a method by which any desirable yeast strain could be made amenable to DNA synthesis studies with [^{3}H]TMP and with BrdUMP. At this stage, there has been no focus on the mechanism of TMP uptake or on genetic characterization of the strains that achieve TMP uptake. The results obtained with a variety of strains indicate that, under suitable conditions (pH, size, and age of inoculum), any yeast strain yields a large percentage of cells able to incorporate TMP in the presence of aminopterin and sulfanilamide. Moreover, it is possible to further select for cells that do not generate petites when grown in the presence of these drugs. The percentage of cells with this ability varies from strain to strain. As a consequence, it is possible to enrich most cultures so as to obtain the most efficient TMP uptake without mutagenesis or having to cross them with TMP uptake mutants.

Both Jannsen (15) and Wickner (21) have reported that TMP uptake strains could incorporate BrdUMP into DNA, as shown by their sensitivity to irradiation by 313 nm of light. In the experiments reported above, we have begun to investigate the incorporation of BrdUMP into yeast DNA to determine whether the analogue would be as useful to study DNA synthesis in yeast as it has proven itself to be in numerous bacterial, fungal, and animal systems (14).

Under our present conditions, it is possible to achieve a density shift of 50 mg/ml in nuclear DNA and 22 mg/ml in mtDNA. This corresponds to 71% BU substitution for nuclear DNA and to 27% substitution for mtDNA, expressed as single-strand labeling. Furthermore, a density shift corresponding to as little as 4% substitution is clearly visible in a CsCl gradient. This constitutes an advantage over density labeling with ^{15}N, for example, which results in a much smaller density shift.

It would be desirable to achieve even higher proportions of substitution by BU, but the toxicity of BrdUMP at concentrations above 85 μg/ml is a limiting factor. Another problem is the degradation of mtDNA at BrdUMP concentrations higher than 25 μg/ml. However, preliminary experiments have shown that this problem can be circumvented by lowering the concentration of TMP in the medium, thus obtaining a high BrdUMP-to-TMP ratio without raising the BrdUMP concentration in the medium above 15 μg/ml. At a concentration lower than 10 μg/ml, BrdUMP is not toxic to the strains that have been tested; thus it is possible to follow growth in the presence of BrdUMP for up to five generations.

Preliminary results indicate that BrdUMP labeling can be used to generate new information on the replication of DNA in yeast, both nuclear and mitochondrial. In their density transfer experiments from heavy (^{15}N) medium to light (^{14}N) medium, Williamson and Fennel (22) found that the buoyant density of mtDNA decreased continuously throughout the transfer. We find, however, two few facts. (i) When a yeast culture is transferred to heavy medium,

mtDNA does not show a progressive increase in buoyant density (for a given BrdUMP/TMP ratio) but attains, after one doubling of the culture, its maximum buoyant density, which will not change even after 24 h of incubation; however, a small amount of mtDNA of intermediate density could have escaped detection. (ii) The proportion of mtDNA that is labeled increases progressively. At the first doubling, only a small fraction of the DNA is labeled and then progressively most of it becomes labeled by the end of the second doubling. Corneo et al. (4) have observed a similar pattern of replication in ^{15}N to ^{14}N density transfer experiments with *S. carlsbergensis*. They reported that heavy mtDNA could still be detected after nuclear DNA had undergone one complete doubling, suggesting a different timing for nuclear and mtDNA synthesis.

Further experiments are necessary to obtain more details on the mechanism of mtDNA replication. These should be greatly facilitated by the use of specific radioactive and density labeling.

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