Induction of mitotic recombination in yeast by starvation for thymine nucleotides

(FdUMP/antifolates/cdc21/pyrimidine metabolism)

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ABSTRACT   The biosynthesis of thymine nucleotides in *Saccharomyces cerevisiae* can be inhibited either by genetic lesions in the structural gene for thymidylate synthetase (*TMP1*) or by drugs that prevent the methylation of dUMP to dTMP. This methylation can be blocked by folate antagonists. We find that 5-fluoro-dUMP (FdUMP) is also an effective inhibitor *in vivo*. Inhibition of dTMP biosynthesis by these three different routes causes thymineless death. In addition to being cytotoxic, we find that FdUMP is highly recombinagenic in yeast but does not induce nuclear gene mutations. Provision of exogenous dTMP eliminates this induced mitotic recombination and cell killing. Similar results were obtained when a thymineless condition was provoked in cells by antifolate drugs or by dTMP deprivation in strains auxotrophic for this nucleotide. These findings show that, in contrast to the situation in prokaryotes, starvation for thymine nucleotides in yeast induces genetic recombination but is not mutagenic.

Cell death caused by growth of thymine auxotrophs in the absence of exogenous thymine was first observed in *Escherichia coli* by Cohen and Barner (1). Subsequent studies by many investigators have revealed that thymine deprivation in prokaryotes can also induce a variety of genetic effects. Thymineless mutagenesis has been demonstrated in *E. coli* (2), *Salmonella typhimurium* (3), *Bacillus subtilis* (4), and bacteriophage T4 (5). Starvation for thymine enhances recombination in λ bacteriophage (6) and in *E. coli* F' merodiploids (7), cures plasmids (8), and induces prophage (9). An episode of thymine deprivation also renders bacteria sensitive to the lethal effects of ultraviolet radiation (10, 11). These various studies were facilitated by the ready availability of bacterial strains that require exogenous thymine because they are mutant at the thymidylate synthetase locus (*thy⁻*).

We know much less about the genetic consequences of thymine starvation or limitation in eukaryotic cells although it has been established that at least some eukaryotes, including yeast (12–15), and murine and human lymphoblasts (16) are susceptible to thymineless death. This paucity of information is due in part to the scarcity of mutant cell strains suitable for such studies.

It is now possible to induce a thymineless condition in cultures of *Saccharomyces cerevisiae* by a variety of methods. Strains defective (12, 15, 17) or conditionally defective (17–19) in thymidylate synthetase have been isolated; these strains are analogous to *thy⁻* bacteria. Where necessary, the yeast mutants can be grown in the presence of exogenous dTMP. The auxotrophic requirement for the nucleotide, rather than thymine or thymidine, derives from the fact that *S. cerevisiae* lacks the enzyme thymidine kinase (20). Thus, thymine nucleotide starvation can be effected by withholding dTMP (12–15) or, in temperature-sensitive strains (*cdc21*), by incubating cells at the restrictive temperature (19).

The methylation of dUMP to yield dTMP requires thymidylate synthetase (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) and the methyl donor N^5,N^{10}-methylenetetrahydrofolate. Drugs that inhibit this enzyme or that limit the supply of reduced folates can abolish dTMP biosynthesis. In yeast, the combination of aminopterin plus sulfonamide is a potent inhibitor (13, 15, 21–25). Cells thus treated require thymidylate, adenine, histidine, and methionine for growth (13, 15). If dTMP is omitted, thymineless death ensues. Bisson and Thorner (17) have shown that FdUMP inhibits yeast thymidylate synthetase *in vitro*. In accord with this latter report, we have found that growth of *S. cerevisiae* strains permeable to dTMP is inhibited by FdUMP and that this treatment can therefore be used to induce thymine nucleotide starvation.

Previous work had shown that dTMP deprivation in yeast was not mutagenic for nuclear genes, but caused mitochondrial point mutations and cytoplasmic petites (14). Similarly, other workers found that thymineless conditions induced mutations in the chloroplast genome of *Chlamydomonas reinhardtii* (26).

In this paper we show that FdUMP is lethal and highly recombinagenic in yeast. Nuclear gene mutations are not induced. The effects of FdUMP can be eliminated by provision of dTMP; this indicates that the cytotoxicity and genetic exchanges are caused by thymine nucleotide starvation. Similar results are obtained when thymidylate deprivation is effected by antifolate drugs or by genetic lesion.

MATERIALS AND METHODS

Strains. The yeast strains used are:

$$D7\text{-}T2 \frac{\alpha\ tup^-\ ade2\text{-}40\ cyh2\ trp5\text{-}12\ ilv1\text{-}92}{a\ tup^-\ ade2\text{-}119\ +\ trp5\text{-}27\ ilv1\text{-}92}$$

$$g430A \frac{\alpha\ cdc21\ +\ +\ ade1\ met1}{a\ cdc21\ lys2\ ade5\ +\ +}$$

$$\frac{+\ ura3 \underset{O}{\quad} hom3\ +\ ilv1\text{-}92\ +}{can1\ +\ +\ arg6\ +\ trp2}.$$

Media. YPD, YPDP, and minimal media have been described (15, 27).

Treatment with FdUMP or Antifolates. Cells of D7-T2 in the logarithmic phase of growth were suspended in YPDP broth (10^6 cells per ml) with or without dTMP (300 µg/ml) and incubated with shaking at 34°C for 30 min (15). They were then transferred to fresh YPDP broth containing either uracil (120 µg/ml) plus FdUMP (500 µg/ml) or aminopterin (100 µg/ml) plus sulfanilamide (5 mg/ml) with or without dTMP (300 µg/ml) and incubated at 34°C with shaking. Uracil was added to prevent the toxic effects of 5-fluorouracil which may have

Abbreviation: mtDNA, mitochondrial DNA.

been present as a contaminant in the commercial preparation of FdUMP. All chemicals were purchased from Sigma.

Detection of Mitotic Events. The diploid strain D7 (28) is heteroallelic at the *ADE2* locus; *ade2-40* causes an absolute requirement for adenine and the formation of red colonies whereas *ade2-119* is leaky and results in pink coloration. These alleles complement so that D7 forms white colonies. Mitotic crossing-over between *ADE2* and the centromere produces red/pink twin sectored colonies. Other genetic events, such as gene conversion, nondisjunction, and forward mutation, result in additional types of colored colonies. There are also two noncomplementing heteroalleles at the *TRP5* locus. Gene conversion at this site is scored by the emergence of tryptophan-independent colonies. In addition, this strain is heterozygous at the *CYH2* locus. Thus, mitotic crossing-over and gene conversion can lead to the expression of the recessive allele for cycloheximide resistance. Strain g430A is heterozygous at a number of loci, including several that flank the centromere of chromosome V. Reciprocal mitotic crossing-over and gene conversion or events leading to hemizygosity can result in the expression of these markers.

RESULTS

Effects of FdUMP on Growth, Viability, and Mitotic Recombination. The biosynthesis of dTMP is catalyzed by thymidylate synthetase (Fig. 1). As shown by Bisson and Thorner (17), yeast thymidylate synthetase can be inhibited *in vitro* by FdUMP. Preliminary tests with haploid strains revealed that the *in vivo* susceptibility to cell killing caused by FdUMP varied among different strains. Those strains capable of taking up
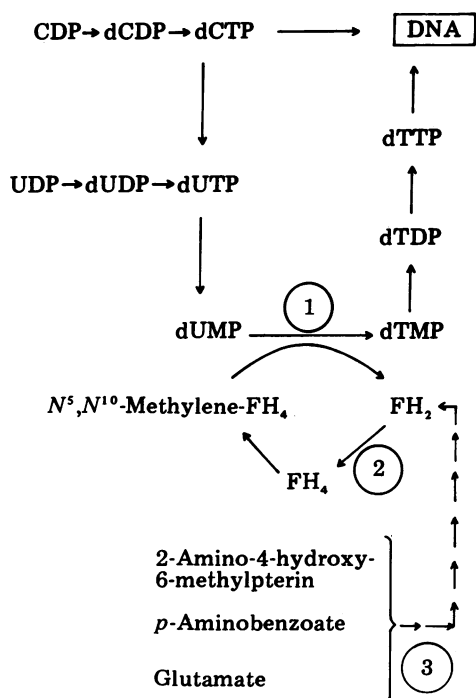

FIG. 1. General scheme for the inhibition of thymidylate biosynthesis. Thymidylate synthetase methylates dUMP to dTMP, and N^5,N^{10}-methylenetetrahydrofolate (FH$_4$, tetrahydrofolate) functions as the methyl donor in reaction 1. *In vitro*, FdUMP inhibits yeast thymidylate synthetase (17). In general, the dihydrofolate (FH$_2$) analogue aminopterin inhibits dihydrofolate reductase (reaction 2) whereas sulfanilamide, an analogue of *p*-aminobenzoate, poisons *de novo* folate synthesis (reaction 3) (29). In yeast, treatment with aminopterin plus sulfanilamide blocks dTMP biosynthesis (13, 15, 21–25), presumably by removing FH$_4$ and so reducing the level of N^5,N^{10}-methylenetetrahydrofolate.

dTMP (*tup⁻*) were sensitive to FdUMP whereas S288C, a wild-type strain (*TUP⁺*), was not. This suggested that dTMP and FdUMP entered the cells by a similar mechanism. In order to optimize the uptake of FdUMP in strain D7 and to monitor the effects of exogenous thymidylate on FdUMP-treated populations, we isolated a derivative of D7 (D7-T2), permeable to dTMP (*tup⁻*), by the method of Little and Haynes (15).

After several hours of growth in YPDP broth containing FdUMP (500 µg/ml), cultures of D7-T2 consisted almost entirely of parent cells with buds of equal size attached. This "dumbbell" morphology is characteristic of cells in which DNA synthesis has been inhibited by genetic lesion (18), by treatment with hydroxyurea (30), or in dTMP auxotrophs starved for thymidylate (12, 15). Shortly after transfer to medium containing the drug, cells began to lose viability (Fig. 2). In general, the cell killing observed resembles the thymineless death kinetics seen in dTMP auxotrophs (14). When the growth medium contained dTMP as well as FdUMP, cells grew at the normal rate.

The killing curve for FdUMP possesses a tail at lower survival levels (Fig. 2). If dTMP were liberated from mitochondrial DNA (mtDNA), which is degraded during thymidylate starvation (14), cell viability could be enhanced, producing the tail on the survival curve. Alternatively, if *TUP⁺* cells unable to take up FdUMP were selected for and grew during the FdUMP treatment, their increase also could account for such a tail. To examine the former possibility, we induced an isolate of D7-T2, lacking mtDNA, by growing the strain for 48 hr in YPD broth containing 100 µg of ethidium bromide per ml. Absence of mtDNA in this isolate [D7-T2 (*rho⁰*)] was confirmed by CsCl gradient centrifugation. This *rho⁰* strain also possesses a tail in its survival curve (Fig. 2); hence, the tail is not dependent on mtDNA. Therefore, we compared the proportions of *TUP⁺* cells present at the beginning and end of FdUMP treatment. Colonies derived from cells that survived 5 hr of growth in the presence of FdUMP were tested for the ability to take up thymidylate by replicating onto YPDP medium containing sulfanilamide, aminopterin, and dTMP. Approximately 4% and 6% of the survivors, for D7-T2 and D7-T2 (*rho⁰*), respectively, were unable to grow on this medium (*TUP⁺*). This indicates that selection for *TUP⁺* cells insensitive to FdUMP cannot account for the tails in the killing curves. The cause of these tails is unknown.
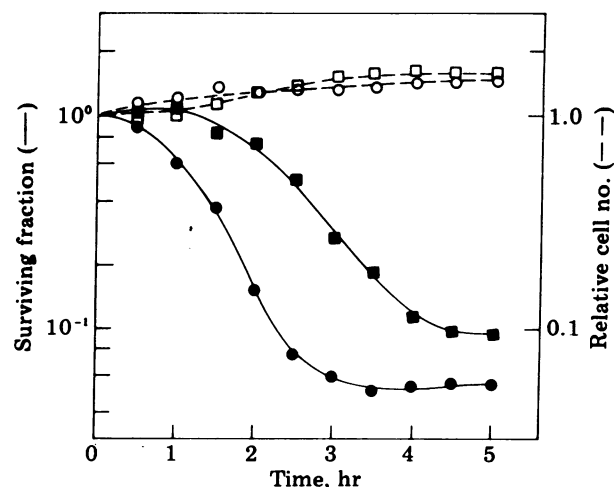

FIG. 2. Effects of FdUMP on growth and viability. D7-T2 and D7-T2 (*rho⁰*) were treated with FdUMP. At 30-min intervals, 1-ml aliquots were withdrawn and washed, and diluted cells were plated on YPD agar medium to determine viability: ●——●, D7-T2; ■——■, D7-T2 (*rho⁰*). Total cell number was determined by Coulter Counter: ○---○, D7-T2; □---□, D7-T2 (*rho⁰*).

The lethality of FdUMP treatment and its reversal by dTMP were demonstrated qualitatively (Fig. 3 *A* and *B*) by a well test (31). In order to determine if mitotic recombination was provoked by FdUMP-induced thymidylate stress, we replicated the plates from the well test onto YPD medium containing cycloheximide. After 4 days of incubation at 34°C, a ring of cycloheximide-resistant colonies emerged in the area of the replicate corresponding to the periphery of the zone of growth inhibition caused by FdUMP (Fig. 3C). However, only a few resistant colonies appeared, distributed randomly, on the replicate of the masterplate that contained dTMP. Because D7-T2 is heterozygous for *cyh2*, the cycloheximide-resistant colonies observed after FdUMP treatment could have arisen not as a consequence of mitotic recombination but as a result of induced monosomy. If this were the case, then resistant cells monosomic for chromosome VII would have lost the homologue containing the wild-type allele *CYH2*. In addition, one of the *trp5* alleles would be missing from these cells because the *TRP5* locus is also situated on chromosome VII and is located between *CYH2* and the centromere. Thus, cycloheximide-resistant derivatives that were of monosomic origin could not undergo gene conversion at *TRP5* because this event requires the presence of both heteroalleles at that locus. Of 70 resistant colonies selected from the ring on the cycloheximide plate, 52 (74%) were capable of UV-induced gene conversion to tryptophan independence. We interpret these data as indicating that the majority of cycloheximide-resistant colonies arose as a result of mitotic recombination rather than nondisjunction. It should be noted that a mitotic crossover between *TRP5* and the centromere of chromosome VII could lead to homozygosity for the *cyh2* allele and for one of the *trp5* alleles. Thus, cycloheximide resistance in the clones incapable of gene conversion could also have resulted from recombination. Clearly then, the aforementioned figure of 74% is a minimal estimate of the proportion of recombinagenic events.
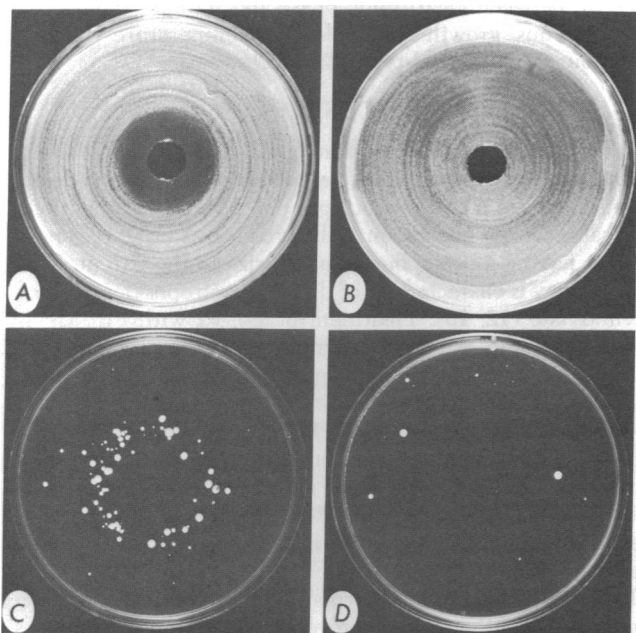
In order to quantitate the induction of mitotic recombination by thymidylate deprivation, cells of D7-T2 in the logarithmic phase of growth were incubated in nutrient broth containing FdUMP. Samples were withdrawn at regular intervals to determine the frequencies of adenine-requiring and of tryptophan-independent cells in the population. Fig. 4 shows that in the absence of exogenous dTMP the frequency of cells that produced colored colonies (*ade⁻*) increased with the time of exposure to the drug and after 6 hr was approximately 250-fold higher than the spontaneous frequency. Similarly, the frequency of tryptophan-independent cells (*trp⁺*) increased 40-fold during this period. Red/pink twin sectors, indicative of mitotic crossing-over, were detected among the induced colored colonies; insufficient numbers precluded the scoring of these events separately. Such twin sectors can occur only if, after the crossover event, both daughter cells formed at the succeeding cell division survive and grow. As viability decreases, the likelihood of both daughter cells surviving also diminishes. As can be seen in Fig. 2, the survival after 2 hr of FdUMP treatment is approximately 15% and, therefore, it is to be expected that few crossovers would be detected.

In the presence of exogenous dTMP, cells grew normally and recombination did not increase significantly above the spontaneous frequencies. Abolition of the FdUMP effects by dTMP is dependent on a 30-min incubation of the cells with thymidylate prior to FdUMP treatment. When FdUMP and dTMP are added concomitantly, some cell clumping occurs and there is a small increase in recombination frequencies.

Reconstruction experiments were performed in which mixtures of D7-T2 and cells derived from spontaneously arising *ade⁻* or *trp⁺* colonies were treated with FdUMP. The survival after a 6-hr exposure to the drug was similar for all three cell types. These results demonstrated that neither an adenine requirement nor tryptophan independence conferred a selective advantage during FdUMP treatment.

FIG. 3. FdUMP-induced expression of cycloheximide resistance. A lawn of D7-T2 cells was spread on YPDP agar medium containing uracil (120 μg/ml) with (*B*) or without (*A*) dTMP (100 μg/ml). FdUMP (500 μg) was added to a center well in each plate. After 24 hr of incubation at 34°C, the plates were replicated onto YPDP medium containing cycloheximide (5 μg/ml). The cycloheximide replicates of the masterplates lacking (*C*) or containing (*D*) dTMP were incubated at 34°C for 4 days.
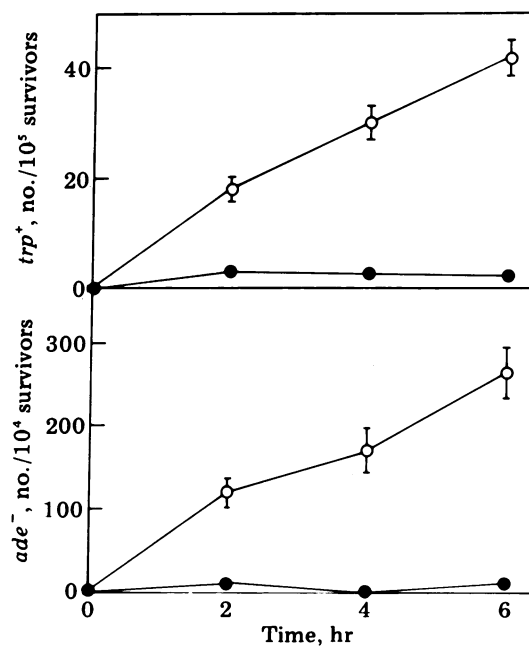

FIG. 4. FdUMP-induced mitotic recombination. D7-T2 was treated with FdUMP in the presence (●) or absence (○) of dTMP. At 2-hr intervals, 1-ml aliquots were withdrawn and washed, then diluted cells were spread on appropriately supplemented minimal agar media. The frequencies of tryptophan-independent (*trp⁺*) colonies and of adenine-requiring (*ade⁻*) colonies were determined after 5 days of incubation at 34°C. Error bars are shown only where the symbols do not overlap them.
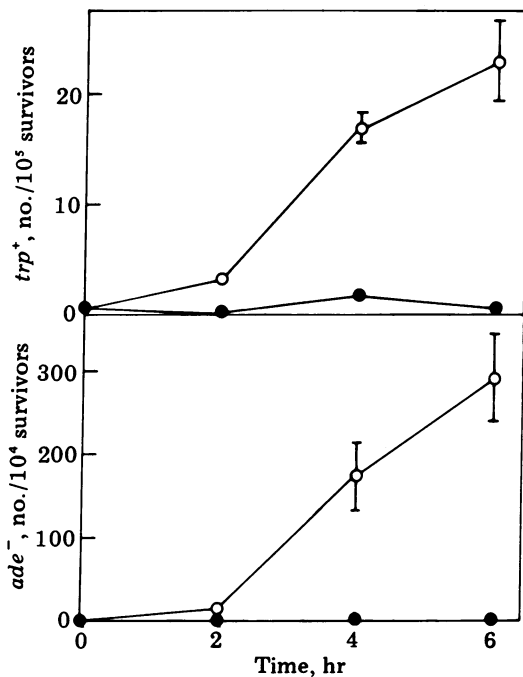
FIG. 5. Antifolate-induced mitotic recombination. Cells of D7-T2 in the logarithmic phase of growth were treated with aminopterin plus sulfanilamide in the presence (●) or absence (○) of dTMP. The sampling procedure is described in the legend to Fig. 4.

Induction of Mitotic Recombination by Antifolates. The *in vitro* activity of thymidylate synthetase from *S. cerevisiae* requires N^5,N^{10}-methylenetetrahydrofolate (17). Concurrent treatment with aminopterin and sulfanilamide inhibits thymidylate biosynthesis (13, 15, 21–25), presumably by depleting endogenous tetrahydrofolate pools (Fig. 1). Incubation of D7-T2 in medium containing these antifolates resulted in the dumbbell morphology observed during inhibition of growth by FdUMP and that is characteristic of thymidylate starvation in dTMP auxotrophs (12, 15). Fig. 5 shows that the thymidylate stress induced by aminopterin plus sulfanilamide induces mitotic recombination. After a 6-hr treatment with the drugs, the frequencies of colored colonies and of gene convertants were, respectively, 300-fold and 25-fold greater than the spontaneous frequencies. Provision of dTMP resulted in normal cell growth and eliminated the increase in recombination.

Table 1.   cdc21-induced expression of recessive markers

Time, hr	Surviving fraction	No. of colonies expressing recessive markers	Total no. of colonies tested	Colonies expressing recessive markers, %
0	1.00	22	2263	0.97
3	1.00	15	1325	1.1
8	0.70	26	995	2.6
20	0.33	44	468	9.4
26	0.19	89	902	10
48	0.02	75	440	17
55	0.01	26	71	37

Cells of g430A in the logarithmic phase of growth, grown overnight at 25°C, were spread on warmed (36°C) YPD agar plates and incubated at 36°C for various periods of time. They were then transferred to 25°C and incubated until colonies appeared. The surviving fractions were determined and the plates were then replicated onto appropriately supplemented minimal media to score for canavanine resistance and for the expression of recessive auxotrophic markers after incubation at 25°C.

Induction of Recessive Homozygosis by *cdc21.* The temperature-sensitive *cdc21* mutation occurs in the structural gene for thymidylate synthetase (17–19). Thus, strains carrying *cdc21* can be starved for dTMP simply by incubating them at 36°C. Game (19) has shown that *cdc21* strains incubated at the restrictive temperature gradually lose viability, and the kinetics of cell killing are similar to those displayed by other dTMP-deprived thymidylate auxotrophs (12, 14). The genetic effects of dTMP starvation in a diploid strain homozygous for *cdc21* were studied by scoring for the expression of recessive markers in cells that survived incubation at 36°C. Strain g430A was initially heterozygous at these loci, several of which flank the centromere of chromosome V. Thus, expression of these alleles could indicate either induced homozygosity via recombination or induced hemizygosity via nondisjunction or deletion. The frequency of expressed recessive markers increased with the time of incubation at 36°C and at 33% survival was 10-fold higher than the control value (Table 1). Of the total events resulting in auxotrophy, 21 involved expression of the *ura3* allele (situated on the left arm of chromosome V). Among these uracil requirers, 12 colonies were found that also were auxotrophic for one or more markers on the right arm of chromosome V. In some instances the expressed alleles were originally in a *trans* configuration (e.g., two colonies were $ura^- arg^- trp^-$), whereas in other cases they were in a *cis* configuration (e.g., five colonies were $can^s ura^- hom^- ilv^-$). The former pattern of expression suggests the occurrence of recombination on both arms of chromosome V, whereas the latter pattern is the type expected for a monosomic event involving chromosome V. The putative monosomic clones could not be examined further by tetrad analysis because they were petites (lacked functional mitochondria) and would not sporulate.

Reconstruction experiments with *cdc21*-uracil auxotrophs demonstrated that selection could have played at most only a minor role in the increase of ura^- events. When *CDC21* strains were grown at 36°C, recombination frequencies remained constant between logarithmic phase and stationary phase of growth. Thus, growth at the elevated temperature was not recombinagenic in wild-type (*CDC21*) strains.

DISCUSSION

Growth of D7-T2 cells in medium containing FdUMP leads to killing, and the loss of viability resembles the thymineless-death kinetics seen in dTMP auxotrophs (14, 15). Cell populations incubated for one generation in the presence of FdUMP consist of cells having a single bud equal in size to the parent. This morphology is characteristic of dTMP auxotrophs deprived of thymidylate (12, 15). Treatment of D7-T2 with FdUMP is recombinagenic. We have determined that the folate antagonists aminopterin and sulfanilamide, which abolish *de novo* thymidylate biosynthesis in *S. cerevisiae* (13, 15, 21–25), also induce mitotic recombination. In addition, incubation of a diploid strain homozygous for the temperature-sensitive mutation *cdc21* [an allele of the gene coding for thymidylate synthetase (17, 19)] at 36°C results in the expression of recessive genetic markers. These lines of evidence suggest that the lethal and recombinagenic effects of FdUMP are a consequence of dTMP starvation. This is corroborated by the demonstration that addition of thymidylate prevents cell killing and eliminates mitotic recombination caused by FdUMP or by antifolates. In summary, depletion of thymine nucleotides, whether induced by drugs or by genetic lesion, is lethal and provokes mitotic recombination in *S. cerevisiae.*

Thymine starvation or limitation is mutagenic in prokaryotes (2–5). In a previous study using a yeast thymidylate auxotroph, dTMP deprivation induced mitochondrial but not nuclear

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mutation (14). During the experiments described here no evidence was obtained for FdUMP- or antifolate-induced reversion of *ilv1-92* nor were we able to detect induced forward mutation at any one of several genes in the biosynthetic pathway of adenine by use of the *ade2 adex* system (32) in a haploid derivative of D7-T2. The fact that yeast is apparently refractory to mutation induction by thymine nucleotide starvation whereas several prokaryotes are readily mutated suggests that eukaryotes and prokaryotes may differ in their response to thymidylate stress.

Various bacterial studies have shown that thymine deprivation results in the accumulation of DNA strand breaks (33–37). DNA strand breakage also occurs during thymidylate starvation of dTMP auxotrophs of yeast (14). This suggests that the recombinagenicity of thymidylate deprivation might be due to the persistence of single-strand gaps in the DNA, a condition that provokes genetic exchange (38, 39).

If DNA strand breaks are involved in thymineless recombination, what then might be the mechanism of induced strand breakage? Recently it has been shown that inhibition of thymidylate synthetase in mammalian cells results in increased levels of dUTP and so enhances misincorporation of uracil into DNA (40). If this is the case in yeast, then the primary molecular event in dTMP-starved cells also might be increased rates of uracil incorporation into DNA. The enzyme uracil-DNA glycosylase (41) functions in the first step of the base excision repair system which removes uracil from DNA (42). This enzyme is present in yeast (W. Crosby, H. Davis, L. Prakash, and D. Hinkle, personal communication). Thus, during early periods of dTMP deprivation DNA-uracil could be excised and replaced by thymine residues derived from the remaining pools of thymine nucleotides in the starving cells. However, when endogenous dTTP pools are exhausted, nascent DNA might then be expected to contain many breaks that resulted from incomplete uracil excision repair. Thus, it is conceivable that uracil incorporation into DNA and its subsequent excision during thymidylate starvation could lead to the formation of DNA strand breaks. To test this hypothesis, mutant strains of yeast defective in various steps of the DNA-uracil excision repair system are required.

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