JOURNAL OF BACTERIOLOGY, Oct. 1977, p. 44-50

Vol. 132, No. 1
Printed in U.S.A.

Thymidine 5'-Monophosphate-Requiring Mutants of *Saccharomyces cerevisiae* Are Deficient in Thymidylate Synthetase

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Received for publication 19 April 1977

Thymidylate synthetase activity was measured in crude extracts of the yeast *Saccharomyces cerevisiae* by a sensitive radiochemical assay. Spontaneous nonconditional mutants auxotrophic for thymidine 5'-monophosphate (*tmp1*) lacked detectable thymidylate synthetase activity in cell-free extracts. In contrast, the parent strains (*tup1*, *-2*, or *-4*), which were permeable to thymidine 5'-monophosphate, contained levels of activity similar to those found in wild-type cells. Specific activity of thymidylate synthetase in crude extracts of normal cells or of cells carrying *tup* mutations was essentially unaffected by the ploidy or mating type of the cells, by the medium used for growth, by the respiratory capacity of the cells, by concentrations of exogenous thymidine 5'-monophosphate as high as 50 μg/ml, or by subsequent removal of thymidine 5'-monophosphate from the medium. Extracts of a strain bearing the temperature-sensitive cell division cycle mutation *cdc21* lacked detectable thymidylate synthetase activity under all conditions tested. Its parent and another mutant (*cdc8*), which arrests with the same terminal phenotype under restrictive conditions, had normal levels of the enzyme. Cells of a temperature-sensitive thymidine 5'-monophosphate auxotroph arrested with a morphology identical to the *cdc21* strain at the nonpermissive temperature and contained demonstrably thermolabile thymidylate synthetase activity. Tetrad analysis and the properties of revertants showed that the thymidylate synthetase defects were a consequence of the same mutation causing, in the auxotrophs, a requirement for thymidine 5'-monophosphate and, in the conditional mutants, temperature sensitivity. Complementation tests indicated that *tmp1* and *cdc21* are the same locus. These results identify *tmp1* as the structural gene for yeast thymidylate synthetase.

Saccharomyces cerevisiae is not permeable to either thymine or thymidine (8) and apparently has no thymidine kinase as well (9). Therefore, one approach for obtaining specific radioactive labeling of yeast deoxyribonucleic acid (DNA) in vivo has been to isolate mutants that require deoxythymidine 5'-monophosphate (dTMP) for growth. Since normal yeast strains are not permeable to dTMP, selection procedures have been devised by several groups that yield mutants permeable to dTMP (23) from which auxotrophs have subsequently been selected (4). The mutations that result in permeability to dTMP define at least four different genetic loci (*tup1-4*) (23). The additional mutations that cause dTMP-permeable strains to require only dTMP for growth all map at the *tmp1* site (4; J. G. Little and J. C. Game, Abstr. Mol. Biol. Yeast Meet., p. 50, 1975). Because of their phenotype, it has been assumed that dTMP auxotrophs are defective in thymidylate synthetase (N^5,N^{10}-methylenetetrahydrofolate:deoxyuridine 5'-monophosphate C-methyltransferase, EC 2.1.1.b). However, for interpreting studies of yeast DNA synthesis utilizing such dTMP auxotrophs, it is important to know whether they are in fact deficient in thymidylate synthetase activity. In this report, we demonstrate that cell-free extracts of dTMP auxotrophs indeed lack detectable thymidylate synthetase activity. In addition, we show that a strain with the cell division cycle mutation *cdc21* (10), whose terminal phenotype at restrictive temperature resembles closely the morphology assumed by dTMP auxotrophs starved for dTMP, contains a defective thymidylate synthetase. Correspondingly, we find that a temperature-sensitive dTMP auxotroph (3) harbors a thermolabile thymidylate synthetase and assumes a morphology at nonpermissive temperature identical to that of the *cdc21* mutant. From these results and genetic data, we conclude that *tmp1*

and *cdc21* represent the same locus, the structural gene for yeast thymidylate synthetase.

MATERIALS AND METHODS

Materials. Reagent chemicals were obtained from the following sources: deoxyuridine-5'-monophosphate (dUMP) and dTMP from P-L Biochemicals; 5-fluoro-dUMP, 5-bromo-dUMP, folic acid, Brij 58, and aminopterin from Sigma Chemical Co.; [5-^{3}H]dUMP and tris(hydroxymethyl)aminomethane (Tris) (enzyme grade) from Schwarz/Mann; dimethyl sulfoxide, platinum oxide, and Norit A from Matheson, Coleman, and Bell; bovine serum albumin (fatty acid poor) from Miles/Pentex; Omnifluor from New England Nuclear; media constituents from Difco; and nitrocellulose filter disks (25-mm diameter, 0.45-μm pore) from Millipore Corp. All other chemicals were reagent grade. Chitosan (deacetyl-chitin) was the gift of Jürgen Weber. *dl*-Tetrahydrofolate (THFA) was prepared by reduction of folic acid with hydrogen gas in the presence of platinum oxide catalyst, substituting an aqueous bicarbonate buffer for the glacial acetic acid used by Rabinowitz and Pricer (17) and followed by chromatography of the product on diethylaminoethyl-cellulose (6). THFA solutions were stored in 0.5 M 2-mercaptoethanol and 0.2 M Tris-hydrochloride (pH 7.5) under vacuum in the dark at 4°C.

Organisms and growth conditions. The genotypes and origins of the strains used are given in Table 1. Cells were grown in medium A containing, in grams per liter: D-glucose, 20; yeast nitrogen base (Difco), 6.7; vitamin-free Casamino Acids (Difco), 2; and adenine, 0.05. This medium was adjusted to pH 5.8 before sterilization. In some experiments, cells were cultivated in YPD, a complex medium, or in SD, a minimal medium, whose compositions have been described (21). For growth of dTMP auxotrophs, media were usually supplemented with 50 μg of dTMP per ml. All solid media contained 2% agar (Difco). Respiratory deficiency was scored as inability to grow on plates of YPE (21), in which 2% ethanol is the major carbon source. Independent spontaneous mutants permeable to dTMP were selected from either X2180-1A or X2180-1B on plates of medium A supplemented with 50 μg of dTMP, 35 μg of aminopterin, and 6 mg of sulfanilamide per ml. Independent spontaneous mutants requiring only dTMP for growth were selected from the permeable strains on the same medium lacking adenine. The rationales for these selections have been discussed in detail elsewhere (4, 23). The particular *tup* and *tmp* mutations in our isolates were determined by their pattern of complementation in diploids resulting from crosses with strains carrying known *tup* and *tmp* mutations (Table 1).

Preparation of cell-free extracts and enzyme assay. Small cultures were inoculated with fresh single-colony isolates of the strains to be tested, grown overnight, and used to inoculate medium (200 ml) in 1-liter side-arm flasks to a cell density of about 5×10^6 cells/ml (approximately 10 Klett units on a Klett-Summerson photoelectric colorimeter with no. 66 red filter). These cultures were grown with vigorous aeration either at 30°C or, for temperature-sensitive strains, at 25°C. When cell density reached 1×10^8 to 2×10^8 cells/ml (180 to 200 Klett units), the cells were collected by centrifugation at 4°C and washed once by suspension in ice-cold buffer A (20%

TABLE 1. *S. cerevisiae strains used*

Strain	Genotype[a]	Source
X2180-1A	a *SUC2 mal gal2 CUP1 rho*$^+$	R. K. Mortimer
X2180-1B	α *SUC2 mal gal2 CUP1 rho*$^+$	R. K. Mortimer
P2180A	a/α *SUC2/SUC2 mal/mal gal2/gal2 CUP1/CUP1 rho*$^+$	This work
B400D	a *SUC2 mal gal2 CUP1 tup1 rho*	This work
B600H	a *SUC2 mal gal2 CUP1 tup4 rho*$^+$	This work
B500B	α *SUC2 mal gal2 CUP1 tup rho*	This work
B500C	α *SUC2 mal gal2 CUP1 tup2 rho*	This work
B410D	a *SUC2 mal gal2 CUP1 tup1 tmp1 rho*	This work
B420D	a *SUC2 mal gal2 CUP1 tup1 tmp1 rho*	This work
B610H	a *SUC2 mal gal2 CUP1 tup4 tmp1 rho*	This work
B510C	α *SUC2 mal gal2 CUP1 tup2 tmp1 rho*	This work
A364A	a *ade1 ade2 his7 gal1 lys2 tyr1 ura1*	L. H. Hartwell
198	a *ade1 ade2 his7 gal1 lys2 tyr1 ura1 cdc8-1*	L. H. Hartwell
H146-2-3	a *ade2 ura1 lys2 cdc21-1*	L. H. Hartwell
211-1aMT2	α *ilv2 typ1 rho*$^+$	M. Brendel
211-1aMT2-1	α *ilv2 typ1 tmp1-1 rho*	M. Brendel
211-1aMT2-10	α *ilv2 typ1 tmp1-10 rho*	M. Brendel
RW108	a *ade2 his4 trp1 tup1-66 rho*	R. B. Wickner
RW109	α *ade2 his4 thr4 leu2 tup1-66 rho*	R. B. Wickner
RW112	a *ade1 ade2 his7 gal1 lys2 tyr1 ura1 tup2-107 rho*$^+$	R. B. Wickner
RW111	α *ade2 his4 gal1 tup2-6 rho*$^+$	R. B. Wickner
RW114	a *ade2 lys2 tup4-55 rho*$^+$	R. B. Wickner
RW115	α *ade2 tup4-55 rho*$^+$	R. B. Wickner
S185	α *ade6 leu1 rho*$^+$	S. Fogel

[a] Genetic nomenclature for *S. cerevisiae* is according to Plischke et al. (16).

glycerol-10 mM 2-mercaptoethanol-10 mM $MgCl_2$-1 mM ethylenediaminetetraacetate [EDTA]-20 mM Tris-hydrochloride, pH 7.4). In most cases, cells were used immediately; however, enzyme activity was stable for at least 1 week in cells stored at −20°C. Washed cells (2 to 3 g, wet weight) were suspended in 2 volumes of buffer A and ruptured by two passages through a chilled French pressure cell at 20,000 lb/in.^{2} Extracts were clarified by centrifugation at 17,000 × *g* for 30 min at 4°C, and the supernatant fluid was assayed for thymidylate synthetase activity by measuring the generation of ^3H_2O from [5-^{3}H]dUMP (13). Reaction was initiated by addition of an appropriate volume of crude extract (1 to 100 μl) to a prewarmed mixture (100 μl) containing [5-^{3}H]dUMP (20 nmol, ~2 × 10^3 cpm/nmol), THFA (140 nmol), formaldehyde (4 μmol), $MgCl_2$ (2 μmol), EDTA (0.2 μmol), 2-mercaptoethanol (20 μmol), Tris-hydrochloride (10 μmol, pH 7.4), and enough buffer A to give a final volume of 200 μl. N^5,N^{10}-methylene-THFA, the true cofactor, is generated spontaneously through reaction of the formaldehyde with the THFA. [A commercial preparation of THFA (Sigma) contained insoluble material and did not work in our system. Moreover, the commercial product was inhibitory to the assay when added along with our preparation of THFA. The inhibitory species did not appear to be *N*-(*p*-aminobenzoyl)-L-glutamate, kindly sent to us by Noel Brawn, Sigma Chemical Co., St. Louis, Mo.] Assay tubes were incubated for 20 min at 25°C in a circulating water bath. The reaction was terminated by chilling and immediate addition of 12% activated charcoal (Norit A) in 0.1 N HCl (0.5 ml). After standing at 0°C for 20 min, the water was collected directly into scintillation vials in a closed manifold system by rapid suction of the quenched reaction mixtures through nitrocellulose filter disks. Each disk was then rinsed once with water (0.5 ml). Bray scintillation fluid (10 ml) was immediately added to each vial, and radioactivity was quantitated in an Isocap 300 liquid scintillation counter (Nuclear-Chicago). The experimental values were corrected by subtracting blank values obtained from incubations without enzyme.

For some experiments thymidylate synthetase activity was measured under the same reaction conditions, using whole cells made permeable to small molecules by treatment with Brij 58 (11), chitosan (12), dimethyl sulfoxide (1), or toluene (20).

Other methods. Protein concentration was determined by the technique of Lowry et al. (14), using bovine serum albumin as a standard.

RESULTS

Detection of thymidylate synthetase activity. Thymidylate synthetase activity was readily followed in crude extracts of yeast cells by the radiochemical method. Under standard conditions, the assay was a linear function both of the amount of protein added (between 0.05 and 1.5 mg) and of time (for at least 30 min), and thus provided an accurate measurement of initial rates. When boiled extract was used, there was no release of tritium whatsoever (Table 2). The presence of boiled extract had no effect on the activity of untreated extracts. Omission of THFA from the assay mixture resulted in very low, but measurable, activity that was largely eliminated by dialysis (Table 2). This suggested that fresh extracts contained the equivalent of 2 to 4 μM THFA. Indeed, in the absence of both added THFA and formaldehyde, only conditions that should have permitted the endogenous generation of the N^5,N^{10}-methylene form of THFA resulted in significant tritium release. For example, a low level of activity was supported by the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine 5'-triphosphate (ATP) with either formate or L-glutamate (Table 2). When only formaldehyde was omitted from the standard assay mixture and formate (100 μM) was substituted, there was again very little activity. Under standard assay conditions, activity was neither augmented nor inhibited by addition of the following compounds,

TABLE 2. *Requirements for thymidylate synthetase activity*

Condition	^{3}H released (pmol/min per mg of protein)
Complete system	
With fresh extract	54
With boiled extract	<0.2
With fresh extract plus boiled extract	55
Complete system	
With fresh extract	51
With dialyzed extract	44
Minus THFA	
With fresh extract	7
With dialyzed extract	2
Complete system	
With fresh extract	43
Plus L-serine and pyridoxal phosphate	46
Plus L-glutamate, ATP, and NADPH	41
Plus formate, ATP, and NADPH	37
Minus THFA and formaldehyde	
With fresh extract	9
Plus L-serine and pyridoxal phosphate	8
Plus L-glutamate, ATP, and NADPH	15
Plus formate, ATP, and NADPH	18
Minus formaldehyde	
Plus formate	8

either alone or in various combinations: L-serine (100 μM), pyridoxal phosphate (100 μM), L-glutamate (100 μM), formate (100 μM), ATP (5 mM), NADPH (1 mM), and K^+ (100 mM) (Table 2).

Although the specific activity for any given strain varied somewhat, depending on the efficiency of cell lysis, specific activities were similar for all strains tested that were presumed to have a functional thymidylate synthetase and were relatively unaffected by the medium used for growth of the cells (Table 3). In addition, the level of activity did not appear to be influenced by the mating type of haploid strains, nor was any gene dosage effect apparent in a diploid. Thymidylate synthetase activity in crude extracts was completely stable to storage for at least 1 week at −20°C. Kinetic measurements indicated that in crude extracts the thymidylate synthetase from normal yeast cells had apparent K_m values for dUMP and for N^5,N^{10}-methylene-THFA of about 5 and 140 μM, respectively. The pH range of 6.5 to 7.5 was optimal for activity.

That the observed tritium release was indeed due to action of thymidylate synthetase was further supported by the effects of halogenated dUMP derivatives known to block thymidylate synthetases from other organisms (18). 5-Fluoro-dUMP completely inhibited the reaction, even at the lowest concentration tested (10 μM). 5-Bromo-dUMP was also an effective inhibitor, with an apparent K_i of about 90 μM estimated from Dixon plots. In addition, product inhibition by dTMP was found, with an apparent K_i of 660 μM.

dTMP auxotrophs lack thymidylate synthetase. Extracts of all of the independently isolated nonconditional mutants that we obtained that required only dTMP for growth lacked detectable thymidylate synthetase activity, regardless of the extent of the dTMP requirement (Table 4). The immediate parent strains had levels of activity similar to those found for wild-type cells. Even when two to three times more protein than usual was assayed, extracts of the strains carrying *tmp* mutations produced no detectable tritium release. Furthermore, when mutant and normal extracts were mixed, the expected level of activity was always observed, indicating that the mutant extracts did not contain an inhibitor of normal thymidylate synthetase.

For dTMP-permeable mutants, the specific activity of thymidylate synthetase was not affected by the concentration of dTMP in the medium used to grow the cells. Furthermore, if these strains were grown up in medium containing dTMP, washed thoroughly by three successive centrifugations and resuspensions in sterile deionized distilled water, and then transferred to medium lacking dTMP and incubated for several generations, no increase in the specific activity of thymidylate synthetase was found. Thus, the immeasurably low level of thymidylate synthetase in the dTMP auxotrophs did not appear to be the result of repression or feedback inhibition of the enzyme due to the dTMP present in the medium used to grow the cells. Such a medium shift was not attempted with the dTMP auxotrophs themselves because we and others (5) have found that they lose viability rapidly in media lacking dTMP. In this regard, however, with dTMP present, variation of other components of the medium still did not yield extracts from the auxotrophs that produced detectable tritium release.

Genetic evidence indicated that the require-

TABLE 3. *Thymidylate synthetase activity in normal yeast cells*

Strain	Mating type	Growth medium	Protein concn (mg/ml)	Sp act (pmol of ^3H released/ min per mg)
X2180-1A	a	YPD	16	24
X2180-1B	α	YPD	20	28
P2180A	a/α	YPD	20	26
A364A	a	YPD	13	27
X2180-1A	a	A	3	54
		SD	3	59
		YPD	6	41
X2180-1B	α	A	4	55
		YPD	9	40
P2180A	a/α	YPD	8	44

TABLE 4. *Thymidylate synthetase activity in dTMP-permeable and dTMP-requiring mutants*

Strain	dTMP requirement[a] (μg/ml)	Protein concn (mg/ml)	Sp act (pmol of ^3H released/ min per mg)
B400D	None	8	31
B600H	None	10	29
B500B	None	5	28
B500C	None	4	33
211-1aMT2	None	10	25
B410D	5	7	<0.2
B420D	5	8	<0.2
B610H	15	5	<0.2
B510C	30	3	<0.2
211-1aMT2-1	15	12	<0.2

[a] Minimum concentration of dTMP required to support growth on agar plates of medium A.

ment for dTMP and the lack of thymidylate synthetase activity were the result of single-site mutations. First, the dTMP auxotrophs were obtained without mutagenesis at a rather high frequency (10^{-8} to 10^{-7}). Second, independent revertants to prototrophy, selected from several different auxotrophs, regained either partial or essentially complete thymidylate synthetase activity (Table 5). Since diploids from backcrosses of dTMP auxotrophs to dTMP-permeable cells were unable to complete the sporulation process, it was not possible to determine whether the requirement for dTMP and the lack of thymidylate synthetase activity cosegregated.

***cdc21* is defective in thymidylate synthetase.** When our dTMP auxotrophs were starved for dTMP, the cells in culture uniformly arrested growth at a characteristic stage in their cell division cycle. This terminal phenotype was that of a cell with a single large bud, in which the nucleus was positioned in the isthmus between the mother and presumptive daughter cell, as revealed by Giemsa staining. This behavior is also typical of two classes of temperature-sensitive cell division cycle mutants, isolated by Hartwell (10), in which DNA synthesis is blocked at the nonpermissive temperature. These are representatives of the *cdc8* and *cdc21* complementation groups. Therefore, we assayed extracts of cells carrying mutations at *cdc8* or *cdc21* and of their parent strain, A364A, grown at the nonrestrictive temperature (25°C) for thymidylate synthetase. The *cdc21* mutant lacked detectable thymidylate synthetase activity at both the permissive and restrictive temperatures (Table 6). Even when buffer A also contained the protease inhibitor phenylmethylsulfonyl fluoride, 0.5 M KCl, 0.1 mM dUMP, or all three compounds, in an attempt to stabilize the enzyme during cell lysis, or when the assay was conducted at 10°C such that the extract never was exposed to a temperature higher than 10°C during processing, no activity was found in extracts of the *cdc21* strain. In contrast, extracts of the *cdc8* mutant and A364A had normal levels of thymidylate synthetase activity (Table 6).

TABLE 5. *Recovery of thymidylate synthetase activity in revertants of dTMP auxotrophs*

Revertant	Sp act[a] (pmol of ^{3}H released/min per 10^9 cells)	Recovery (% of normal)
B600H	12.0	100
B611H-R1	1.3	11
B611H-R2	1.5	13
B611H-R3	1.2	10
B400D	9.7	100
B420D-R1	0.4	4
B420D-R2	8.1	84
B420D-R3	0.3	3

[a] As measured in whole cells permeabilized with Brij 58; see Materials and Methods.

TABLE 6. *Thymidylate synthetase activity of temperature-sensitive mutants*

Strain	Initial rate (pmol of ^{3}H released/min per mg of protein)			
	10°C	25°C	30°C	37°C
A364A	8.0	27.4		28.5
198 (*cdc8*)		18.0		16.0
H146-2-3 (*cdc21*)	<0.2	<0.2	<0.2	<0.2
H146-2-3-9BR (ts^r)		51.0		58.0
H146-2-3-2CR (ts^r)		21.0		16.0
H146-2-3-3AR (ts^r)		8.4		9.3
211-1aMT2		19.6	27.0	31.0
211-1aMT2-10 (*tmp1-10*)		2.0	0.5	<0.2

When extract from the *cdc21* strain was mixed with extract from normal cells, the expected level of activity was observed, showing that the mutant extract did not contain an inhibitor of normal thymidylate synthetase. Furthermore, whereas enzyme activity was readily measured at either 25 or 37°C in whole cells of A364A made permeable by a number of different chemical treatments, by none of these methods could thymidylate synthetase activity be detected in permeabilized cells carrying the *cdc21* mutation.

That the temperature sensitivity and the lack of thymidylate synthetase activity in *cdc21* cells were due to a single-site mutation was supported by genetic evidence. First, in a backcross to a wild-type cell (S185), the two characteristics cosegregated in all tetrads that yielded four viable spores (8 out of 10 asci dissected). Second, several independently isolated temperature-resistant revertants regained essentially normal thymidylate synthetase activity (Table 6).

***tmp1* and *cdc21* represent the structural gene for thymidylate synthetase.** The *cdc21-1* mutation was the only allele at that locus obtained in Hartwell's original selection that showed "first-cycle arrest" (10). If the temperature sensitivity and cell division cycle-specific blockade in cells carrying the *cdc21* mutation are a manifestation only of its exceedingly labile thymidylate synthetase, then a strain with a temperature-sensitive requirement for dTMP should harbor a temperature-sensitive thymidylate synthetase and should behave like the *cdc21* mutant when placed at the nonpermis-

sive temperature. Such a temperature-sensitive dTMP auxotroph, 211-1aMT2-10 (*tmp1-10*), was selected by Brendel and Fäth (3). We found that this strain indeed contains low but demonstrably thermolabile thymidylate synthetase activity (Table 6). Moreover, when shifted to restrictive conditions, this strain arrested growth with a uniform terminal morphology identical to that observed with the *cdc21* mutant.

Diploids resulting from crosses of our nonconditional dTMP auxotrophs or the temperature-sensitive dTMP auxotroph with the *cdc21* strain are still temperature sensitive for growth and are unable to sporulate. This lack of complementation strongly implies that *tmp1* and *cdc21* are the same genetic locus.

DISCUSSION

The specific activity of thymidylate synthetase in extracts of *S. cerevisiae* (Table 3) is about one-tenth that reported for extracts of *Escherichia coli* B (19). This is perhaps surprising, since S phase in rapidly growing yeast cells is approximately equal to the generation time for a rapidly proliferating bacterial culture, about 25 min (25). During this period, the yeast cell must duplicate three to four times as much DNA (P. A. Whitney and B. D. Hall, Abstr. Mol. Biol. Yeast Meet., p. 45, 1975), with a higher adenine-thymine content (60 to 64%), than does *E. coli*. On the other hand, the yeast cell may be accumulating precursors for DNA synthesis throughout the remainder of the cell division cycle and during the preceding G1 period. It should also be pointed out that the specific activities obtained in this work represent average values for cells in asynchronous culture. It is quite possible that the level of thymidylate synthetase increases markedly just before and during S phase, as has been observed in some other systems (2). Indeed, in experiments in which significantly higher specific activities than usual were observed (Table 3), the results appeared to be due to selective breakage of budded cells, which presumably represent the most actively growing members of the population.

Every one of the dTMP auxotrophs that we isolated (10 out of 10) lacked detectable thymidylate synthetase activity in vitro, as did the dTMP auxotroph sent to us by M. Brendel (Table 4). Perhaps the selection conditions are too stringent to recover partial or leaky mutants. Unlike Brendel and his co-workers, who used mutagenesis to obtain such mutants (4), we were able to select these auxotrophs spontaneously. Meiotic segregation patterns and the properties of revertants indicate that the lack of thymidylate synthetase activity and the requirement for dTMP in these auxotrophs are the result of single-site mutations.

Our measurements also show that cells carrying either of the temperature-sensitive mutations, *cdc21-1* or *tmp1-10*, contain an exceedingly labile thymidylate synthetase (Table 6). These findings support and extend the recent observation of Game that growth of a double mutant (*tup cdc21*) is spared at the nonpermissive temperature by the addition of exogenous dTMP (7). In addition to our complementation tests, which suggest that the *cdc21* and *tmp1* mutations are allelic, recent results have shown that *cdc21* and *tmp1* map at similar positions: on chromosome XV, loosely linked to *ade2* (7, 24). From our in vitro assays and the genetic findings, it seems reasonable to conclude that *tmp1* and *cdc21* are the same locus and define the structural gene for yeast thymidylate synthetase.

Although none of our dTMP auxotrophs complement each other or *cdc21*, it has been reported that one previously isolated allele, *tmp1-6*, would complement *cdc21* in heteroallelic diploids (7). Such an exceptional case could be due to intragenic complementation if the yeast enzyme were an oligomeric protein, as has been demonstrated for thymidylate synthetases from other organisms. For example, the thymidylate synthetase from *Lactobacillus casei* is a dimer of two identical 35,000-molecular-weight subunits (18).

The *cdc21* mutant has a phenotype characteristic of a chromosome elongation mutant; that is, *cdc21* cells halt DNA synthesis abruptly after a shift to a restrictive temperature (10). This effect could be explained if the pool of deoxythymidine 5'-triphosphate available for DNA synthesis in this mutant were quite low, even under permissive conditions. This may be the case, since *cdc21* cells rapidly stop synthesis of both nuclear and mitochondrial DNA after a shift to a restrictive temperature (10, 15). (This observation and our finding that respiratory capacity does not seem to influence the level of thymidylate synthetase [Table 4] indicate that there may be only a single thymidylate synthetase in yeast cells.) On the other hand, it has been suggested that the deoxycytidine 5'-monophosphate hydroxymethylase induced upon infection of *E. coli* by bacteriophage T4, an enzyme that performs a function similar to that of thymidylate synthetase, is an integral part of a "replication complex" required for T4 DNA synthesis in vivo (22). Perhaps yeast thymidylate synthetase also has such a dual role.

ACKNOWLEDGMENTS

We would like to thank Anne Delk and Jesse Rabinowitz for helping us to prepare THFA and for other invaluable assistance.

This investigation was supported in part by Public Health Service research grant GM21841 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Adams, B. G.** 1972. Method for decryptification of α-glucosidase in yeast with DMSO. Anal. Biochem. 45:137-146.
2. **Blakeley, R. L.** 1969. The biochemistry of folic acid and related pteridines, p. 231-260. Wiley-Interscience, New York.
3. **Brendel, M., and W. W. Fäth.** 1974. Isolation and characterization of mutants of *Saccharomyces cerevisiae* auxotrophic and conditionally auxotrophic for 5'-dTMP. Z. Naturforsch. 29c:733-738.
4. **Brendel, M., W. W. Fäth, and W. Laskowski.** 1975. Isolation and characterization of mutants of *Saccharomyces cerevisiae* able to grow after inhibition of dTMP synthesis, p. 287-294. *In* D. M. Prescott (ed.), Methods in cell biology, vol. XI. Academic Press Inc., New York.
5. **Brendel, M., and U. G. Langjahr.** 1974. Thymine-less death in a strain of *Saccharomyces cerevisiae* auxotrophic for deoxythymidine 5'-monophosphate. Mol. Gen. Genet. 131:351-358.
6. **Curthoys, N. P., J. M. Scott, and J. C. Rabinowitz.** 1972. Folate coenzymes of *Clostridium acidi-urici*. J. Biol. Chem. 247:1959-1964.
7. **Game, J. C.** 1976. Yeast cell-cycle mutant *cdc* 21 is a temperature-sensitive thymidylate auxotroph. Mol. Gen. Genet. 146:313-315.
8. **Grenson, M.** 1969. The utilization of exogenous pyrimidines and the recycling of uridine 5'-phosphate derivatives in *Saccharomyces cerevisiae*, as studied by means of mutants affected in pyrimidine uptake and metabolism. Eur. J. Biochem. 11:249-260.
9. **Grivell, A. R., and J. F. Jackson.** 1968. Thymidine kinase: evidence for its absence from *Neurospora crassa* and some other microorganisms and the relevance of this to the specific labelling of deoxyribonucleic acid. J. Gen. Microbiol. 54:307-317.
10. **Hartwell, L. H.** 1973. Three additional genes required for deoxyribonucleic acid synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. 115:966-974.
11. **Hereford, L. M., and L. H. Hartwell.** 1971. Defective DNA synthesis in permeable yeast mutants. Nature (London) New Biol. 234:171-172.
12. **Jaspers, E. T. A., K. Christianse, and J. van Steveninck.** 1975. An improved method for the preparation of yeast enzymes *in situ*. Biochem. Biophys. Res. Commun. 65:1434-1439.
13. **Kammen, H. O.** 1966. A rapid assay for thymidylate synthetase. Anal. Biochem. 17:553-556.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
15. **Newlon, C. S., and W. L. Fangman.** 1975. Mitochondrial DNA synthesis in cell cycle mutants of *Saccharomyces cerevisiae*. Cell 5:423-428.
16. **Plischke, M. E., R. C. Von Borstel, R. K. Mortimer, and W. E. Cohn.** 1976. Genetic markers and associated gene products in *Saccharomyces cerevisiae*, p. 765-832. *In* G. D. Fasman (ed.), Handbook of biochemistry and molecular biology, 3rd ed. CRC Press, Cleveland, Ohio.
17. **Rabinowitz, J. C., and W. E. Pricer.** 1957. An enzymatic method for the determination of formic acid. J. Biol. Chem. 229:321-328.
18. **Rader, J. I., and F. M. Huennekens.** 1973. Folate coenzyme-mediated transfer of one-carbon groups, p. 197-223. *In* P. D. Boyer (ed.), The enzymes, 3rd ed., vol. IX. Academic Press Inc., New York.
19. **Roodman, S. T., and G. R. Greenberg.** 1971. A temperature-sensitive *thy* mutant blocked in the synthesis of thymidylate synthetase. J. Biol. Chem. 246:2609-2617.
20. **Serrano, R., J. M. Gancedo, and C. Gancedo.** 1973. Assay of yeast enzymes *in situ*: a potential tool in regulation studies. Eur. J. Biochem. 34:479-482.
21. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1974. Methods in yeast genetics: a laboratory manual. Appendix A. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Tomich, P. K., C.-S. Chiu, M. G. Wovcha, and G. R. Greenberg.** 1974. Evidence for a complex regulating the *in vivo* activities of early enzymes induced by bacteriophage T4. J. Biol. Chem. 249:7613-7622.
23. **Wickner, R. B.** 1975. Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine 5'-monophosphate into DNA *in vivo*, p. 295-302. *In* D. M. Prescott (ed.), Methods in cell biology, vol. XI. Academic Press Inc., New York.
24. **Wickner, R. B., and M. J. Leibowitz.** 1976. Two chromosomal genes required for killing expression in killer strains of *Saccharomyces cerevisiae*. Genetics 82:429-442.
25. **Williamson, D. H.** 1966. Nuclear events in synchronously dividing yeast cultures, p. 81-101. *In* I. L. Cameron and G. M. Padilla (ed.), Cell synchrony. Academic Press Inc., New York.