THYMIDINE UTILIZATION BY *TUT* MUTANTS AND FACILE CLONING OF MUTANT ALLELES BY PLASMID CONVERSION IN *S. CEREVISIAE*

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Manuscript received May 1, 1986
Revised copy accepted July 25, 1986

ABSTRACT

Plasmid pJM81 contains a Herpes simplex virus thymidine kinase (*TK*) gene that is expressed in yeast. Cells containing the plasmid utilize thymidine (TdR) and the analogue 5-bromodeoxyuridine (BUdR) for specific incorporation into DNA. TdR auxotrophs, harboring plasmid pJM81 and a mutation in the yeast gene *TMP1* require high concentrations of TdR (300 μg/ml) to support normal growth rates and the wild-type mitochondrial genome (ρ^+) cannot be maintained. We have identified a yeast gene, *TUT1*, in which recessive mutations allow efficient utilization of lower concentrations of TdR. Strains containing the mutations *tmp1* and *tut1*, as well as plasmid pJM81, form colonies at 2 μg/ml TdR, grow at nearly normal rates and maintain the ρ^+ genome at 50 μg/ml TdR. These strains can be used to radiolabel DNA specifically and to synchronize DNA replication by TdR starvation. In addition, the substitution of BUdR for TdR allows the selective killing of DNA-synthesizing cells by 310-nm irradiation and allows the separation of replicated and unreplicated forms of DNA by CsCl equilibrium density banding. We also describe a unique, generally applicable system for cloning mutant alleles that exploits the fact that Tk^+ yeast cells are sensitive to 5-fluorodeoxyuridine (FUdR) and that gene conversions can occur between a yeast chromosome and a *TK*-containing plasmid.

THE study of DNA replication in prokaryotes and eukaryotes has been facilitated greatly by the ability to label DNA specifically. Exogenous thymidine (TdR), converted to thymidine monophosphate (dTMP) by thymidine kinase (TK), enters the *de novo* pathway of thymidine triphosphate (dTTP) formation. However, yeast and other fungi do not have TK activity (GRIVELL and JACKSON 1968), and studies of DNA replication *in vivo* have been hampered because the labeling of DNA with radioactive TdR or with dense nucleoside analogues, such as 5-bromodeoxyuridine (BUdR) is precluded [see FANGMAN and ZAKIAN (1981) for a review]. This limitation has been bypassed with *tup* mutations which make yeast cells permeable to dTMP (BRENDEL and FÄTH 1974; WICKNER 1974; LITTLE and HAYNES 1979; BISSON and THORNER 1982), or, more recently, by employing high copy number plasmids containing an active *TK* gene from the Herpes simplex virus (MCNEIL and FRIESEN 1981).

TABLE 1

***S. cerevisiae* strains**

Strain	Relevant genotype	Plasmid
151	*MATa tmp1-6 tut1-1 leu2-3,112 cyh2 can1*	pJM81
121[a]	*MATα tmp1-6 tup leu2-3,112 ρ⁻*	pJM81
131	*MATa leu2-3,112*	
132	*MATα leu2-3,112*	
153	*MATa tmp1-6 tut1-2 leu2-3,112 cyh2 can1*	pJM81
184	*MATa tmp1-6 tut1-2 bar1 cdc7-4 cyh2 can1 leu2-3,112*	pJM81
124	*MATa tmp1-6 leu2-3,112 can1 ρ⁻*	pJM81
123	*MATa tmp1-6 tut1-1 can1 cyh2 leu2-3,112*	pJM81
236	*MATa leu2-3,112 cdc7-1*	
222	*MATa trp1-289 ura3-52 leu2-3,112 cdc7::URA3/MATα ura3-52 leu2-3,112*	

Strains 131, 132, 151, 153, 184, 236 and 222 are congenic with strain A364a (HARTWELL 1967).

[a] The same as strain YTK2 (MCNEIL and FRIESEN 1981) containing pJM81.

If the strain also has a mutation in the yeast *TMP1* gene coding thymidylate synthase (LITTLE and HAYNES 1979), the cell is auxotrophic for dTMP or TdR, respectively. Significant drawbacks to these two systems are that the strains produce respiration-incompetent cells (ρ^-) at a high frequency (BRENDEL and FÄTH 1974; WICKNER 1974; LITTLE and HAYNES 1979; BISSON and THORNER 1982), and high concentrations of dTMP or TdR are needed for normal rates of growth. Because of the physiological alterations associated with ρ^- yeast (DUJON 1981), and because most *tup* mutations result in pleiotropic phenotypes (WICKNER 1974; BISSON and THORNER 1982), we decided to generate ρ^+ strains that can be labeled efficiently with thymidine *in vivo* but do not exhibit physiological differences from wild-type yeast. In addition, we describe a procedure in which mutant alleles can be easily cloned *in vivo* by selecting for loss of the *TK* gene on a plasmid that occurs by a conversion event with a chromosomal gene containing the desired allele.

MATERIALS AND METHODS

Strains and media: All yeast strains are *S. cerevisiae* and are listed in Table 1. *E. coli* strain JF1754 (MCNEIL and FRIESEN 1981) was used for the propagation of yeast-*E. coli* shuttle vectors. Yeast media used were YEPD and Y complete (ZAKIAN, BREWER and FANGMAN 1979) or dropout media (SCLAFANI and FANGMAN 1984a) that were supplemented with TdR at 100 μg/ml for routine use. Cell number determination employed Coulter Counter model Zf with a 70 μm orifice. DNA transformation employed either the sphaeroplast or whole cell methods (HINNEN, HICKS and FINK 1978; ITO *et al.* 1983). Strain construction used either tetrad or random spore analysis (MORTIMER and HAWTHORNE 1975) in which TdR was included in all media at 100 μg/ml. Petites (ρ^-) were detected by an inability to grow on glycerol as a carbon source using YEPG agar (MORTIMER and HAWTHORNE 1975; SCLAFANI and FANGMAN 1984a) supplemented with TdR at 100 μg/ml.

Cell synchronization and radiolabeling: Cultures of *cdc7* strains were synchronized with α-factor and temperature shifts (ZAKIAN, BREWER and FANGMAN 1979; SCLAFANI and FANGMAN 1984b). For steady-state labeling of the DNA, [*methyl*-^{3}H]TdR (Amer-

sham; TRK.418 40 Ci/mmol) was used in complete medium at 20 μCi/ml plus unlabeled TdR at 100 μg/ml. Cells were filtered from this medium and were placed into the same medium with unlabeled BUdR substituted for the TdR, but maintaining [*methyl*-^{3}H]-TdR at 20 μCi/ml. Cells were processed and DNA was isolated as described (ZAKIAN, BREWER and FANGMAN 1979). Samples were adjusted to 54.5% CsCl in 0.1 M EDTA and 0.1 M Tris-HCl, pH 8.0 (n^{25} = 1.4034), and were centrifuged to equilibrium in a VTi65 rotor for 20 hr at 45,000 rpm at 20°. Gradient fractions were collected from the bottom of the tube onto Whatman 3MM filters and were processed as described (LARK and WECHSLER 1975). All operations with BUdR-containing cultures were performed in subdued yellow light.

In experiments using [^{14}C]adenine, [8-^{14}C]adenine (Amersham; CFA.348 50 mCi/mmol) was used at 1 μCi/ml, 2.5 μg/ml. Samples were taken and radioactivity in DNA or DNA was determined (ZAKIAN, BREWER and FANGMAN 1979).

Long-wave irradiation: Samples of cultures to be irradiated were placed in ice-cold sterile H_2O in a glass Petri dish and were kept on ice during irradiation. A 40-watt G.E. fluorescent sunlamp, at a distance of 5 cm, provided the irradiation. A 1-cm-deep solution of TdR (1 mg/ml) in a glass Petri dish was placed above the Petri dish containing the cells as a shortwave UV filter. Cells were irradiated for 40 min.

Allele recovery: Strains (Table 1) were transformed with pRS282 (see Figure 5), a plasmid containing the Herpes *TK* gene. The TK-containing cells were sensitive to FUdR (Fud^s) at concentrations of 50 μg/ml on Y complete minus leucine dropout agar. The omission of leucine from the medium provides selection for the plasmid containing the *LEU2* gene. Strains without plasmid or with the vector lacking the *TK* gene alone were completely resistant to FudR (Fud^R) at these concentrations. At 23°, Fud^R colonies appear after 3–4 days of incubation.

Plasmids were isolated from yeast DNA preparations by selection for Amp^R in *E. coli* and were subjected to routine analysis by DNA restriction enzymes and agarose gel electrophoresis (SCLAFANI and FANGMAN 1984a,b). Ligations and *E. coli* transformations were performed as described previously (SCLAFANI and FANGMAN 1984a). Plasmid pRS282 (see Figure 5) was prepared from pRS277 that contains the 2.2-kb *LEU2 Sal*I-*Xho*I fragment ligated into the *Sal*I site of pBR322, the 0.85-kb *Eco*RI-*Hin*dIII *ARS1* and 2.0-kb *Hin*dIII-*Bam*HI *CEN3* fragments from plasmid YCp41 (CLARKE and CARBON 1980) ligated into the *Eco*RI-*Bam*HI sites of pBR322, and the 4.5-kb *Bgl*II fragment containing the *CDC7* gene (PATTERSON *et al.* 1986) ligated into the *Bam*HI site of the pBR322 DNA sequences.

The 3.4-kb *Bam*HI DNA fragment, containing *HIS3* and the *TK* gene, was obtained from plasmid pRC4-TK$^+$ (SCLAFANI and FANGMAN 1984a) by digestion with *Bam*HI endonuclease and purification as described (SCLAFANI and FANGMAN 1984a). Ligation into pRS277 was performed and recombinants were selected as Amp^R His$^+$ in strain JF1754 (KUNZ *et al.* 1980; MCNEIL and FRIESEN 1981) to obtain plasmid pRS282 (see Figure 5). The 3.4-kb DNA fragment can be used as a *Pvu*II fragment (MCNEIL and FREISEN 1981) or as a *Bam*HI fragment (SCLAFANI and FANGMAN 1984a) for insertion into cloned DNA sequences.

RESULTS

The *TUT1* gene: Strain YTK2 (MCNEIL and FRIESEN 1981) contains the *tmp1-6* mutation that prevents *de novo* synthesis of dTMP from dUMP, a *tup1* mutation that permits utilization of dTMP from the medium, and the Herpes *TK* gene that provides both thymidine kinase and dTMP kinase activities (SCLAFANI and FANGMAN 1984a) on a multicopy plasmid, pJM81. Expression of the *TK* gene apparently depends on a promoter in the adjacent *HIS3* sequences found on the plasmid (MCNEIL and FRIESEN 1981). YTK2 can utilize TdR or dTMP in the medium; however, it is ρ^- and cannot grow on a nonfermentable

carbon source such as glycerol, and it requires a high concentration of TdR (200 μg/ml) for growth. We have found this strain difficult to grow and to use for cell synchronization. Starting with a *TUP* derivative of YTK2 (strain 124), mutants were selected that could grow with TdR at 2 μg/ml. These mutants appeared at a frequency of $1–5 \times 10^{-5}$. We refer to this phenotype as Tut$^-$ (TdR utilization). When *tmp1* Tut$^-$ strain 123 containing pJM81, here called *tmp1* Tut$^-$ (*TK*), was crossed to *tmp1* Tut$^+$ strain 124, the diploids were Tut$^+$; therefore, the *tut* mutation is recessive. However, these diploids were ρ^- and could not be sporulated. Therefore, the *tmp1* Tut$^-$ (*TK*) strain was crossed to *TMP1* Tut$^+$ strain 131. Since the *tmp1-6* and *tut* mutations are recessive, this diploid was Tmp$^+$ Tut$^+$ ρ^+. Selection for the pJM81 plasmid was achieved by growing the diploids in the absence of leucine, since pJM81 also contained the *LEU2* gene, and both haploid parents were *leu2* (Table 1). The diploids were sporulated and tetrads were dissected. In asci with four viable spores (40%), two spores were Tmp$^-$ (required TdR) and two spores were Tmp$^+$ as expected, since the *tmp1-6* mutation is a single lesion (BRENDEL and FÄTH 1974). In the most frequent type of tetrad (12 of 15), one Tmp$^-$ spore was Tut$^-$ (required 2 μg/ml TdR) and ρ^+, and one Tmp$^-$ spore was Tut$^+$ (required 100 μg/ml TdR) and ρ^-. In two tetrads (2 of 15) both Tmp$^-$ spores were Tut$^-$ ρ^+, and in one tetrad (1 of 15) both Tmp$^-$ spores were Tut$^+$ ρ^-. The frequencies are those expected if *tmp1-6* and the *tut* mutation segregate independently. The *tut* mutation cannot be on the TK plasmid, pJM81, because the Tut$^-$ phenotype did not segregate 4:0 (LIVINGSTON 1977). The tetrad data indicate that the *TUT* gene is probably unlinked to the *TMP1* gene and that a single mutation in it allows both growth with 2 μg/ml thymidine and maintenance of the mitochondrial genome (ρ^+). Consistent with this conclusion, a random spore analysis showed that 59% (32 of 54) of all Tmp$^-$ spores were ρ^+ and Tut$^-$. Ten independent spontaneous Tut$^-$ mutants were isolated; none complements the original *tut* mutation. Therefore, these comprise a single complementation group, *TUT1*.

The frequency of mitochondrial petites (ρ^-) was determined after growing cells on solid medium containing glucose and different concentrations of TdR. At ≥50 μg/ml TdR, colonies of a *tmp1-6 tut1-2* (*TK*) strain contained 80–90% ρ^+ cells. At lower concentrations of TdR, the fraction of ρ^- cells increases; for example, at 10 μg/ml only 1–5% were ρ^+.

Growth and DNA labeling: The following experiments were conducted using the *tut1-2* allele in strains that are congenic with strain A364a (Table 1). The *tmp1-6 tut1-2* (*TK*) strain 153 growing in complete medium with TdR at 100 μg/ml had a doubling time of 2.5 hr at 30°, compared to 2.0 hr for a *TMP1* strain. When [^{3}H]Tdr (100 μ/ml, 20 μCi/ml) was included in the medium for five doublings, 4×10^7 cells yielded about 2×10^4 dpm in acid-precipitable, alkali-stable material. When the culture was filtered and placed in fresh medium with unlabeled TdR, no increase in the amount of incorporated label was observed for two doubling times, suggesting that the internal pool of thymine nucleotides is not large. High specific radioactivities can also be used effectively for shorter labelings. For example, when an exponential

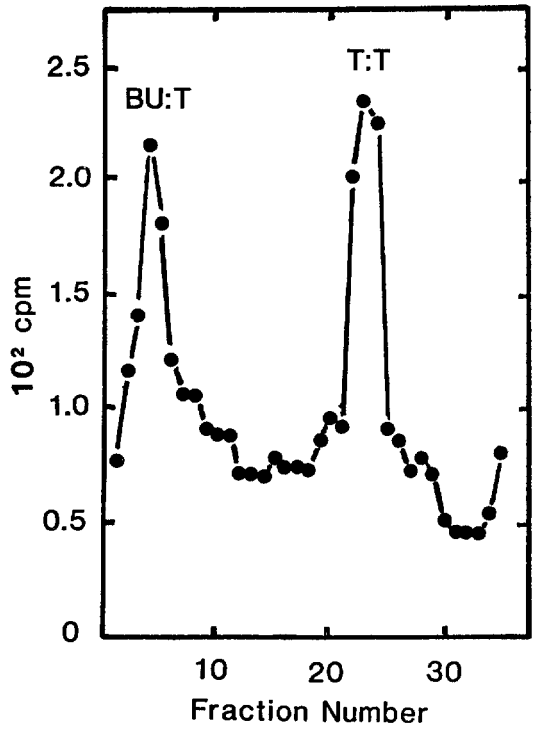

FIGURE 1.—CsCl gradient of BUdR-labeled yeast DNA. Exponential cells of strain 153 (2 × 10^6/ml) growing in Y complete medium (ZAKIAN, BREWER and FANGMAN 1979) containing [^{3}H]-Tdr at 20 μCi/ml (100 μg/ml) were filtered, washed and resuspended in the same medium with BUdR (100 μg/ml) in place of TdR and [^{3}H]TdR at 20 μCi/ml. Cells were grown for 0.5 doubling time (75 min) at 30°, filtered, and washed with ice-cold SCE, and DNA was isolated (ZAKIAN, BREWER and FANGMAN 1979). Samples were centrifuged to equilibrium in CsCl density gradients and were processed (MATERIALS AND METHODS).

phase culture was filtered and placed in medium containing [^{3}H]TdR at 10 μg/ml and 10 μCi/ml for 1 hr (~0.4 doublings), about 1.5 × 10^4 dpm were incorporated by 4 × 10^7 cells. When the 1-hr labeling period was followed by the addition of unlabeled TdR at 300 μg/ml, the amount of incorporated label stayed constant for the remainder of the experiment (3 hr). It should be noted that, when cells are grown in TdR at <50 μg/ml, respiratory-deficient mutants (ρ^-) accumulate rapidly.

Thymidine analogues and culture synchronization: Since the *tmp*1 *tut*1 (*TK*) strains require TdR, 100% substitution with halogenated nucleotides can be achieved using halogenated deoxyribonucleosides in the medium. An asynchronous culture of strain 153 was radiolabeled with [^{3}H]TdR (20 μCi/ml, 100 μg/ml) for about five doublings. The culture was filtered, washed and resuspended in fresh medium containing BUdR (100 μg/ml) and [^{3}H]TdR (20 μCi/ml). After 0.5 doublings, a band appeared in CsCl gradients at the density expected for fully hybrid (BU-T) DNA (Figure 1). The percentage of ^{3}H label in BU-T DNA was about 51%, reasonably close to the 58.6% expected at 0.5 doublings (SCLAFANI and FANGMAN 1984b).

To synchronize yeast cells in the S phase (ZAKIAN, BREWER and FANGMAN 1979) the *cdc7 tmp*1 *tut*1 (*TK*) strain 184 was constructed. A culture that had been labeled with [^{3}H]Tdr (20 μCi/ml, 50 μg/ml) for five doublings at 23° was arrested with α-factor for one doubling time (4.5 hr). Ninety percent of the cells were unbudded after arrest, compared to 30% in the initial asynchronous culture. The cells were filtered, washed and placed in fresh medium containing BUdR (100 μg/ml) and [*methyl*-^{3}H]TdR at 20 μCi/ml. The medium had been prewarmed to 36°, the restrictive temperature for the *cdc7* mutant. After incubation at 36° for 0.5 doubling times (2.25 hr) the cells had become 85% budded, with uniformly large buds as expected for the *cdc7* mutant (CULOTTI and HARTWELL 1971; HARTWELL *et al.* 1973); the culture was then placed at 23°. At this time <5% of the DNA was hybrid (BU-T) in density (data not shown) as expected, since the initiation of nuclear DNA replication is blocked in *cdc7* mutants under these conditions (HARTWELL 1973). (Mitochondrial DNA, about 10% of the total, had little effect on the [^{3}H]DNA profiles in the CsCl gradients. Initially observed as a light shoulder on the peak of nuclear DNA, it shifted toward hybrid density during the *cdc7* arrest at 36°.) The amount of BU-T DNA increased at a constant rate during the S phase, reaching a value of 60% after 100 min at 23° (Figure 2). Therefore, *tmp1 tut*1 (*TK*) strains can be used for the density-labeling of DNA with BUdR in both asynchronous and synchronous cultures.

Selective killing of BUdR-containing cells: Long-term incorporation of BUdR into DNA resulted in a decrease in cell viability (GREER 1960). Yeast *tmp1 tut1* (*TK*) cells (strain 153) in logarithmic growth were filtered, washed and resuspended in complete medium containing BUdR (100 μg/ml) in place of TdR. Cells were diluted in TdR medium and were spread on YEPD + TdR agar; viability decreased with increasing time in the BUdR medium (Figure 3). In addition, only the cells grown in BUdR were sensitive to long-wave UV light (~310 nm). After irradiation (MATERIALS AND METHODS), viability was reduced an additional 16-fold for cells exposed to BUdR for one doubling time, and 150-fold for cells exposed to BUdR for 2.0 doubling times. The former cells have one strand of DNA substituted with BUdR, whereas most of the latter (~75%) have both strands substituted with BUdR. Therefore, irradiation of cultures incubated with BUdR can be used selectively to kill cells synthesizing DNA.

TdR starvation and culture synchronization: All *tmp1* mutants have defects in thymidylate synthase and are allelic to *cdc21* mutants (GAME 1976; BISSON and THORNER 1977). *tmp*1 *tup* auxotrophs starved for dTMP undergo thymineless death (BARCLAY and LITTLE 1978; LITTLE and HAYNES 1979); however, the *tmp1 tut1* (*TK*) strains described here could be starved for at least two doubling times before a significant decrease in cell viability was detected (Figure 3). Furthermore, the fraction of cells forming petite (ρ^-) colonies remained fairly constant during the period of starvation: when a culture was starved for ~6.5 doublings, viability was reduced to 0.25, but the fraction of ρ^- cells increased only from 10 to 16%. During starvation, most of the cells (90%) arrested as large budded cells and continued to grow in size, as expected

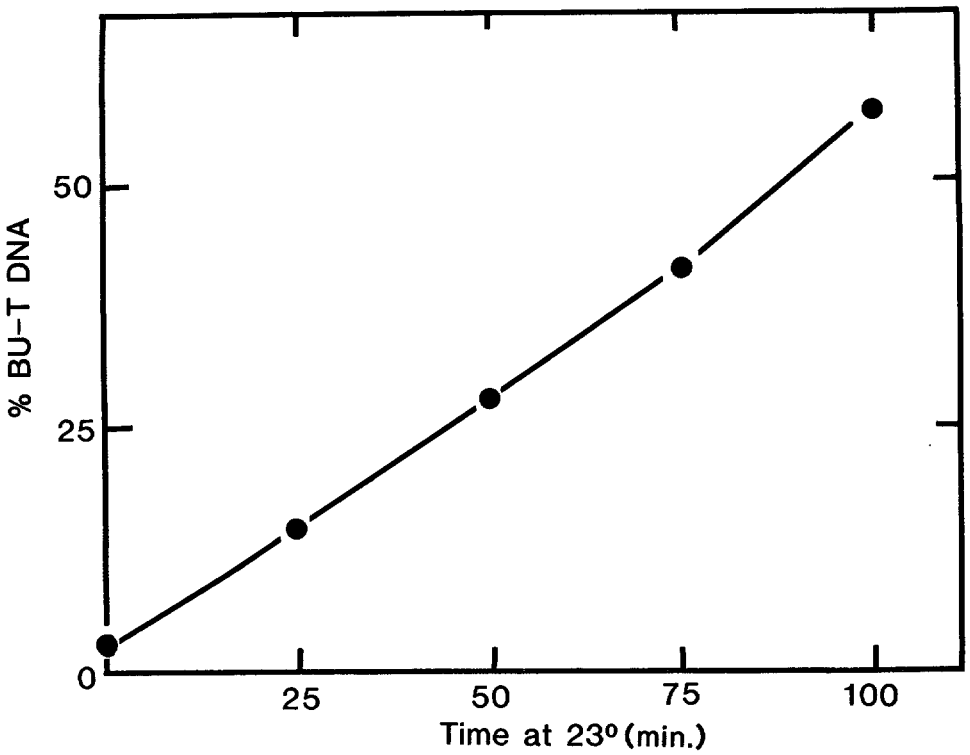

FIGURE 2.—Production of hybrid (BU-T) DNA by synchronized S phase cells. Cultures of strain 184 were synchronized at the G_1/S boundary, as described in the text, and were permitted to synthesize DNA in the presence of BUdR. Samples were processed and quantitated as described in MATERIALS AND METHODS.

from observations with *cdc21* mutants (HARTWELL 1973). Therefore, starvation for TdR for one generation creates a population of cells arrested at the G_1 phase-S phase boundary; the block is reversible since 100% of the cells remain viable. The rate of killing of *tmp1 tup* cells during thymidine starvation has been reported to vary with cell density and genetic background (BARCLAY and LITTLE 1978; KUNZ *et al.* 1980). However, we have not detected an effect of cell density on cell viability or ρ^- formation during TdR starvation over a range of 5×10^5 to 5×10^6 cells/ml in complete medium. (Cells reach stationary phase at $\sim 2 \times 10^7$ cells/ml in complete medium + TdR).

Cultures of *tmp1 tut1* (*TK*) cells (strain 184) were incubated with α-factor, then were removed from α-factor by filtration and placed in complete medium lacking TdR. Viability remained constant for a length of time equivalent to at least two doubling times after the release from α-factor (data not shown). Therefore, a viable population of yeast cells synchronized at the beginning of S phase was easily obtained. Radiolabeling experiments indicate that DNA synthesis, but not RNA synthesis, was terminated during TdR starvation and that a round of DNA synthesis began immediately upon the addition of TdR or BUdR (Figure 4). Since the DNA synthesis block was completely reversible and did not require temperature shifts, the sequence of action of a *CDC* gene

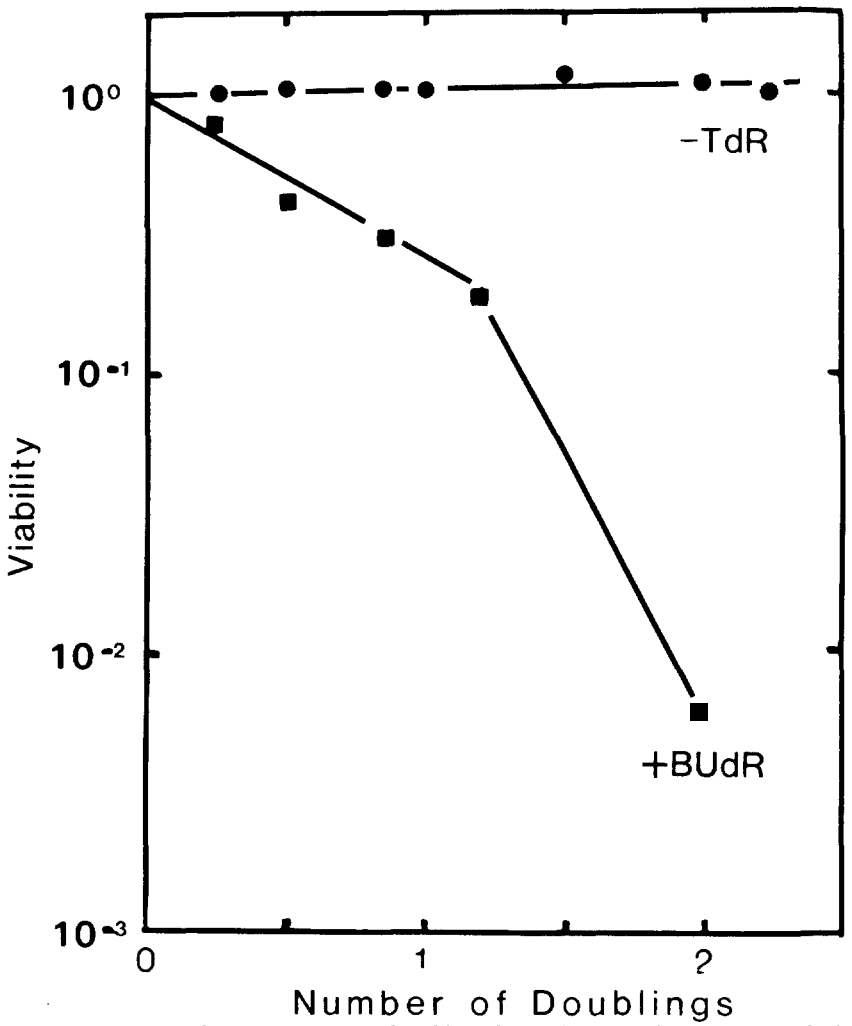

FIGURE 3.—Viability of *tut1 tmp1* (*TK*) cells subjected to TdR starvation or BudR substitution. Exponential cells (1 × 10^6/ml) of strain 153 growing in Y complete medium containing 100 μg/ml TdR were filtered and resuspended either in medium without TdR or medium in which BUdR (100 μg/ml) was substituted at $t = 0$. Samples were spread on YEPD + TdR (100 μg/ml) agar. ● — ●, starved for TdR; ■ — ■, grown in BUdR. (One doubling time in normal medium equals 2.5 hr.)

product relative to the time of DNA synthesis could be determined by reciprocal shift experiments (HARTWELL 1976; MOIR and BOTSTEIN 1982) using *tmp1 tut1 cdc*^{ts} or *tmp1 tut1 cdc*^{cs} strains.

Allele recovery: To recover mutant alleles from a yeast chromosome, we have used the Herpes *TK* gene in the following scheme. The Herpes *TK* gene was inserted in or near the gene of interest present on an *ARS1* LEU2 CEN3 single-copy plasmid, and the plasmid was transformed into a *leu2* strain that contains a mutant allele. The *TK* gene confers 5-fluorodeoxyuridine (FUdR) sensitivity on yeast because of the enzymatic conversion of FUdR to FdUMP,

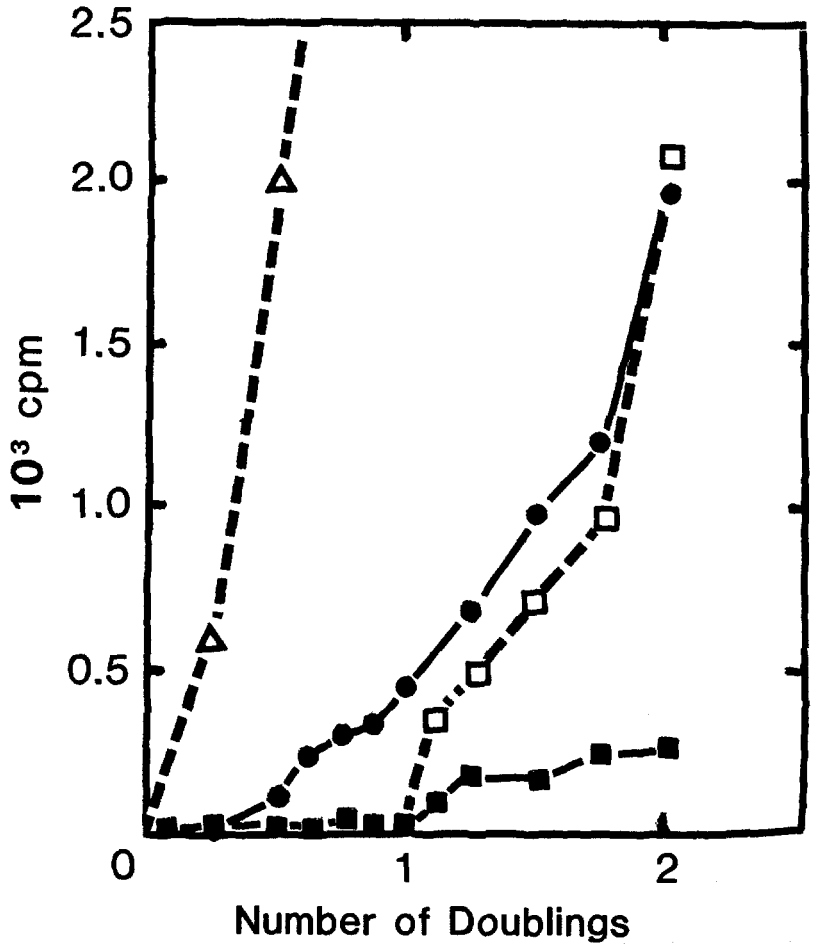

FIGURE 4.—Radiolabeling of *tmp1 tut1* (*TK*) cells with [^{14}C]adenine after synchronization by TdR starvation. Exponential cells of strain 184 (2×10^6/ml) were filtered, washed and resuspended in Y complete medium with [^{14}C]adenine at 1 μCi/ml, (2.5 μg/ml) at 0 time. Half of the culture was incubated at 23°; samples were taken and processed to determine radioactivity in DNA or RNA. RNA (Δ – – Δ) and DNA (■ — ■) in the culture without TdR; DNA (● — ●) in the culture with TdR; □ – – □, a portion of the culture without TdR to which TdR was added after one doubling. RNA synthesis in the culture with TdR was the same as in the culture without TdR (data not shown). (A doubling time equals 4.5 hr.)

a potent inhibitor of yeast thymidylate synthase (BISSON and THORNER 1977, 1982); *i.e.*, Tk^+ cells are FUdR-sensitive (Fud^s) and Tk^- cells are FUdR-resistant (Fud^R). By selecting for maintenance of the plasmid (Leu^+) but against the *TK* gene (Fud^R), we expected to obtain plasmids that had lost the *TK* gene by gene conversion events involving the chromosomal allele of the gene of interest. Some fraction of plasmids will harbor the mutant allele, with the frequency depending on the distance in the gene between the mutation and the *TK* gene insertion. Subsequently, the plasmid can be recovered in *E. coli* and subjected to analysis.

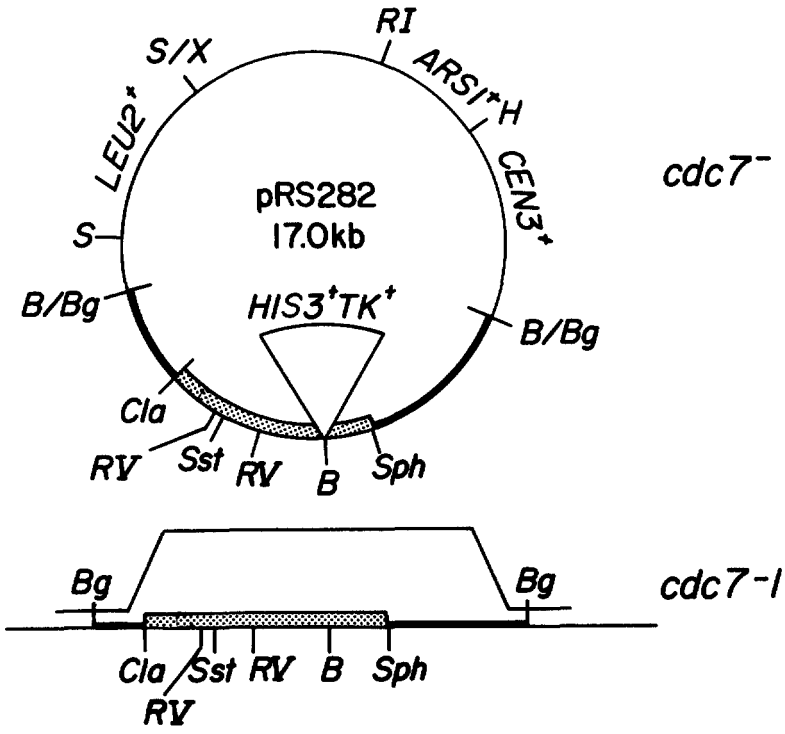

FIGURE 5.—Conversion event between plasmid pRS282 and yeast chromosome *IV*. Restriction maps of pRS282 and the homologous region on chromosome *IV* containing the *cdc7-1* allele are depicted. The region that is to be converted on the plasmid is shown as a line between the two DNAs. The stippled region represents *cdc7* coding sequences; the triangle represents the 3.4-kb *HIS3 TK* insertion. Sequences not involved directly in the recombination event are depicted with a thin black line. The position of the yeast *LEU2*, *CEN3* and *ARS1* sequences is also shown. Symbols for DNA restriction endonuclease sites are *S* = *Sal*I; *S/X* = *Sal*I/*Xho*I hybrid; *RI* = *Eco*RI; *H* = *Hin*dIII; *B/Bg* = *Bam*HI/*Bgl*II hybrid; *B* = *Bam*HI; *Cla* = *Cla*I; *RV* = *EcoRV; Sst* = *Sst*I; *Sph* = *Sph*I, *Bg* = *Bgl*II.

We have tested this scheme with the *cdc7-1* allele of gene *CDC7*. Plasmid pRS282 contains a 3.4-kb *Bam*HI *HIS3 TK* fragment inserted into the *Bam*HI site of the *CDC7* gene (PATTERSON *et al.* 1986) on plasmid pRS277 (Figure 5) and, therefore, is LEU2 *cdc7::HIS3 TK*. Strain 236 (*leu2 cdc7-1*) harboring pRS282 is therefore Fud^s Leu$^+$ and Tsm$^-$ (temperature-sensitive). Fud^R Leu$^+$ colonies appeared at a frequency of 2×10^{-5} and fell into four phenotypic classes (Table 2). The four classes are interpreted as follows.

Class I colonies were Tsm$^-$, but were stably Leu$^+$. They probably represent events in which the *leu2* locus on the chromosome was converted to *LEU2*, with subsequent loss of the plasmid. Class II, III and IV colonies all had an unstable Leu$^+$ phenotype (5–10% Leu$^-$ cells after ten generations of nonselective growth) indicating retention of the *leu2* chromosome locus and a *LEU2* plasmid. Class II colonies were unstably Tsm$^+$, with Tsm$^+$ cosegregating with Leu$^+$ consistent with their representing conversions that resulted in loss of the *TK* gene and restoration of the wild-type *CDC7* gene on the plasmid. Class III

TABLE 2

Phenotype of derivatives of strain 236

Vector or class	Fud	Leu$^+$	Tsm	PAP	Frequency (%)
None	R	NA	−	−	NA
pRS277	R	U	+	NA	NA
pRS282	S	U	−	+	NA
Class I	R	S	−	−	10
Class II	R	U	+	NA	26
Class III	R	U	−	+	10
Class IV	R	U	−	−	54

Vectors pRS277 (*CDC7*) and pRS282 (*cdc7::HIS3TK*) are described in the text. Fud, Resistant (R) or sensitive (S); Leu$^+$ phenotype, unstable (U) or stable (S); Tsm, growth (+) or no growth (−) at 36° on YEPD plates; PAP, production (+) or no production (−) of Tsm$^+$ papillae at high frequency (100 times background) on colonies replica-plated to 36°; NA, not applicable. The frequency at which each of the classes I–IV arises is shown in the last column.

colonies were Tsm$^-$, but gave rise to Tsm$^+$ papillae, as did colonies containing the original plasmid pRS282. These colonies probably represent events in which the *TK* gene became defective by mutation but the plasmid retained most of the original DNA sequences. Tsm$^+$ papillation occurred because the plasmid contained the wild-type sequences corresponding to the *cdc7-1* mutational change that, by recombination, could generate a wild-type chromosomal *CDC7* gene. Class IV colonies were stably Tsm$^-$, suggesting that they represent events in which the interrupted *CDC7* gene on the plasmid was converted to *cdc7-1*. To test this interpretation we recovered several of these plasmids in *E. coli* by selecting for Amp^R after transformation. DNA restriction enzyme analysis indicated that the 3.4-kb *HIS3 TK* insert had been completely removed, restoring a DNA fragment of the expected size (data not shown). Most important, by placing a putative *cdc7-1* plasmid into a yeast cell heterozygous for a *cdc7* insertion mutation (*CDC7/cdc7::URA3*; R. SCLAFANI, J. ROSAMOND and W. L. FANGMAN, unpublished results), we have shown that the plasmid, pRS285, can rescue *cdc7*::URA3 haploid spores from an otherwise lethal phenotype (see below).

On sporulation of the diploid strain 222 (*trp1/TRP1, ura3/ura3, leu2/leu2, cdc7::URA3/CDC7*), the only viable spores produced were Ura$^-$ Leu$^-$. The 222 cells transformed with the centromere plasmid pRS277 (*LEU2, CDC7*) on sporulation gave many spores that were Leu$^+$. All of the Ura$^+$ Leu$^+$ spores were Tsm$^+$; that is, as expected, the plasmid-rescued spores that would otherwise have been unable to form colonies because of the deficiency for the *CDC7* gene product. When plasmid pRS285 (*LEU2*, putatively *cdc7-1*) replaced pRS277, all of the Ura$^+$ Leu$^+$ spores were Tsm$^-$, consistent with the *cdc7-1* allele being on the plasmid. The cosegregation frequency of the Trp$^+$ and Ura$^+$ phenotypes in all three sporulations confirmed that the normally unlinked *TRP1* and *URA3* loci were closely linked (~3 cM) as expected for *URA3* inserted at the *CDC7* locus (*CDC7* and *TRP1* are closely linked). Since plasmid

pRS285 complemented the *cdc7::URA3* insertion mutation at 23°, but not at 36°, it must contain the *cdc7-1* mutation.

DISCUSSION

The *tmp1 tut1* (*TK*) yeast strains described here will be useful for both DNA replication and cell-cycle studies. The *tut1* mutations are recessive lesions that permit *tmp1* (*TK*) yeast to utilize TdR efficiently. Perhaps the permeability of *tut1* yeast cells to deoxynucleosides is increased compared to that of wild-type cells that are relatively impermeable (GRENSON 1969). All ten *tut* mutations isolated so far lie in one complementation group, *tut1*. It is easy to construct strains that are *tut1 tmp* (*TK*) by standard genetic techniques (MORTIMER and HAWTHORNE 1975) by supplementing the media with TdR. From pedigree analysis, we estimate that more than 90% of the cells contain the 2-μm plasmid vector pJM81 with the *TK* gene, because the yeast *tmp1* mutation selects for its maintenance. Unlike *tmp1 tup1* strains, *tmp1 tut1* (*TK*) strains are not inhibited by concentrations of up to 10 mg/ml of the appropriate precursor (dTMP or TdR). We did not detect an effect even when solid TdR was placed on agar plate spread with *tmp1 tut1* (*TK*) cells. The minimal TdR concentration required for maximal growth rate and ρ^+ maintenance is 50 μg/ml. We routinely use Tdr at 100 μg/ml in minimal or nutrient media. Under these conditions, approximately 90% of the cells in cultures of these strains are ρ^+.

*tmp*1 *tut*1 cells with a single copy of the *TK* gene, on a *CEN* plasmid or integrated into a yeast chromosome, can also grow with TdR in the medium; however, the growth rate is reduced and the frequency of ρ^- cells is increased, even with high concentrations of TdR. The small fraction of ρ^- cells in cultures of cells containing a high copy *TK* plasmid may arise in those cells that, because of variations in copy number of 2-μm plasmids (FUTCHER and COX 1984), reach a low level of *TK* enzyme activity. It may be possible to select mutants or construct *TK* plasmids in which these properties are eliminated.

The useful properties of *tmp1 tut1* (*TK*) yeast can be summarized as follows:

1. DNA can be radiolabeled with TdR or density labeled with the nucleoside analogue BUdR. Unlike the corresponding 5′-nucleotide monophosphates, both are readily available and are inexpensive. The internal pool of thymidine precursors appears to be small, allowing rapid labeling of DNA.

2. Cells can be synchronized in the S phase by blocking DNA synthesis through removal of TdR from the medium. The block is completely reversible, an important property for experiments in which events of the cell cycle are to be ordered (HARTWELL 1976; MOIR and BOTSTEIN 1982). During TdR starvation, DNA synthesis is blocked but RNA synthesis continues (Figure 4). The unbalanced growth condition only slowly results in cell death. In contrast, *tmp tup* strains are (inexplicably) sensitive to dTMP deprivation at low cell densities (BARCLAY and LITTLE 1978; LITTLE and HAYNES 1979).

3. The stability of the mitochondrial genome is high (~90% ρ^+ cells), even under nonselective conditions (glucose medium). Although the stability is not as high as with *TMP1* cells (~99%), it is high enough to allow cell synchronization and mitochondrial DNA replication studies. This property of *tmp1 tut1*

(*TK*) yeast is a significant improvement over the *tmp1 tup* strains previously described (LITTLE and HAYNES 1979).

4. After long-term labeling with BUdR, irradiation with long-wave UV light results in a 10^3-fold decrease in cell viability. Cells not synthesizing DNA [*e.g.*, α-factor arrested cells (HEREFORD and HARTWELL 1974)] are selectively resistant. This property can be exploited to isolate DNA replication mutants as described for prokaryotes (BONHOEFFER and SCHALLER 1965; CARL 1970). (Mutant isolation is currently under way in the laboratory of R.A.S.)

We have used Herpes *TK* plasmids for gene transplacements (ROTHSTEIN 1983; data not shown) and to efficiently transfer mutant alleles from a yeast chromosomal gene to a wild-type copy on a plasmid by gene conversion. For the latter manipulation, the important feature of the *TK* gene is that cells containing it can be selected against with 5-fluorodeoxyuridine (FUdR); the *TK* enzyme activity converts FUdR to dFUMP, a potent inhibitor of yeast thymidylate synthase (BISSON and THORNER 1977, 1982). As expected, cells that lack the *TK* gene are resistant to FUdR. For recovery of chromosomal mutant alleles, the functional *TK* gene is inserted within (or near to) the corresponding cloned wild-type yeast gene on a plasmid containing *LEU2* as a selectable marker. Selection for *LEU2* (minus leucine) and against *TK* (plus FUdR) yields plasmid gene convertants in which the *TK* insert is replaced by material from the chromosomal gene that often includes the mutant allele. All that is required is a single DNA restriction site in or near the gene of interest into which the 3.4-kb *HIS3 TK Bam*HI (SCLAFANI and FANGMAN 1984a) or *Pvu*II (MCNEIL and FRIESEN 1981) DNA restriction fragment can be inserted. Therefore, we believe the method is simpler than procedures requiring gap filling of plasmids (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981) or transplacement (ROTHSTEIN 1983) and will be of general use.

The successful expression of the Herpes *TK* gene in yeast achieved by MCNEIL and FRIESEN (1981) was a breakthrough for DNA specific-labeling in this organism. We believe that the *tmp1 tut1* (*TK*) strains described here provide useful improvements for the utilization of TdR and its analogues that can be adapted to a variety of experimental designs.

We thank BRIAN MCNEIL and JAMES FRIESEN for strains YTK2, JF1754 and plasmid pJM81, and NANCY GAMBLE and KAREN SHEA for superb secretarial assistance. The work was supported by a National Institutes of Health Research Service Award (to R.A.S.) and Public Health Service grants GM35078 (to R.A.S.) and GM18926 (to W.L.F.).

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Communicating editor: E. JONES