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The *GTS1* Gene, Which Contains a Gly-Thr Repeat, Affects the Timing of Budding and Cell Size of the Yeast *Saccharomyces cerevisiae*

KAZUHIRO MITSUI, SO-ICHI YAGUCHI, AND KUNIO TSURUGI*

Department of Biochemistry, Yamanashi Medical University, Tamaho, Nakakoma, Yamanashi 409-38, Japan

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A gene with an open reading frame encoding a protein of 417 amino acid residues with a Gly-Thr repeat was isolated from the yeast *Saccharomyces cerevisiae* by using synthetic oligonucleotides encoding three Gly-Thr dimers as probes. The deduced amino acid sequence showed partial homology to the clock-affecting gene, *per*, of *Drosophila melanogaster* in the regions including the GT repeat. The function of the gene, named *GTS1*, was examined by characterizing the phenotypes of transformants with different copy numbers of the *GTS1* gene produced either by inactivating the *GTS1* gene by gene disruption (TMΔgts1) or by transformation with multicopy plasmid pPER119 (TMpGTS1). They grew at similar rates during the exponential growth phase, but the lag phases were shorter for TMΔgts1 and longer for TMpGTS1 cells than that for the wild type. Analyses of their cell cycle parameters using synchronized cells revealed that the unbudding period changed as a function of gene dosage; that is, the periods of TMΔgts1 and TMpGTS1 were about 20% shorter and longer, respectively, than that of the wild-type. Another significant change in the transformants was detected in the distribution of the cell size. The mean cell volume of the TMΔgts1 cells in the unbudded period (single cells) was 27% smaller than that of single wild-type cells, whereas that of single TMpGTS1 cells was 48% larger. Furthermore, in the temperature-sensitive *cdc4* mutant, the *GTS1* gene affected the timing of budding at the restrictive temperature. Thus, the *GTS1* gene product appears to modulate the timing of budding to obtain an appropriate cell size independent of the DNA replication cycle.

Among the genes known to affect biological clocks, the *period* gene (*per*) of *Drosophila melanogaster* (24, 37) and the *frequency* gene (*frq*) of *Neurospora crassa* (31) were the first to be isolated and sequenced. Characteristically, the amino acid sequences deduced from the nucleotide sequences of both genes each contained a Gly-Thr/Ser repeat which is shared by proteoglycans comprising a polysaccharide attachment site (31, 37, 43). Although the GT repeats are polymorphic in length and in sequence among the species of *Drosophila* (10, 11, 16, 34, 35, 48, 51), the presence of the GT repeat in the clock-affecting genes from evolutionarily remote species, *Drosophila* and *Neurospora* species, implies that this sequence plays an important role in most, if not all, clock-affecting proteins.

The nature of *per* mutant phenotypes suggested that the gene product is an integral component of the circadian clock: mutations either shorten (*per*^{s}), lengthen (*per*^{1}), or abolish (*per*^{0}) not only the period of circadian rhythms (19) but also the rhythm with a much shorter period during the male courtship song (26, 52). Furthermore, the period of the circadian rhythm of *D. melanogaster* changes depending upon the gene dose (4, 12, 45). Thus, a gene dose-dependent control and pleiotropic mutant phenotypes affecting various rhythmic biological processes were shown to be present in some mutant alleles of a typical clock gene.

Biological clocks are considered to be ubiquitous in eukaryotic and prokaryotic organisms (for a review, see reference 18). Even in yeasts, a few lines of evidence for the presence of a biological clock have been reported, although no clock-related genes have yet been designated. Edmunds and coinvestigators demonstrated that the yeast *Saccharomyces cerevisiae* shows a circadian rhythm in cell division activity when cultured at low temperatures, although only about one-half of the cells divided during any one light-dark cycle and the period often varied considerably (14). Later, they reported that the cell division cycle is regulated by a circadian oscillator by using the unicellular algal flagellate *Euglena gracilis* (1). Furthermore, Hartwell suggested the presence of an intracellular timer in yeasts which regulates the timing of bud initiation, since the temperature-sensitive mutant *cdc4* initiates buds periodically independent of DNA replication and cell division at restrictive temperatures (20, 21).

In this study, we isolated a potential clock gene from the yeast *S. cerevisiae* using synthetic oligonucleotides encoding three GT repeats. A positive clone contained an open reading frame (ORF) which encodes a protein of 417 amino acid residues and which has some homology to other clock genes in the primary sequence and potential protein motifs. The function of the putative clock gene was examined by characterizing the phenotypes of transformants with different copy numbers of the gene produced either by inactivating the gene by gene disruption or by transformation with a multicopy plasmid harboring the gene. Analyses of the cell cycle parameters of the transformants by using synchronized cells showed that the unbudding period and cell sizes increased as a function of the gene dosage.

MATERIALS AND METHODS

Yeast strains and media. Strains of the yeast *S. cerevisiae* IFO10151 (*MAT***a** *ade2 his3-532 trp1-289 ura3-1,2 can*^{r} Inos$^-$) and IFO10094 (*MAT*α *cdc4 can1 his2 hom3 leu2-1 lys9 met1 pet8 rad4 trp2 ura3*) were obtained from the Institute for Fermentation (Osaka, Japan) and used for transformation.

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* Corresponding author. Mailing address: Department of Biochemistry, Yamanashi Medical University, 1110 Shimokato, Tamaho, Nakakoma, Yamanashi 409-38, Japan. Phone: 0552-73-1111. Fax: 0552-73-6784.

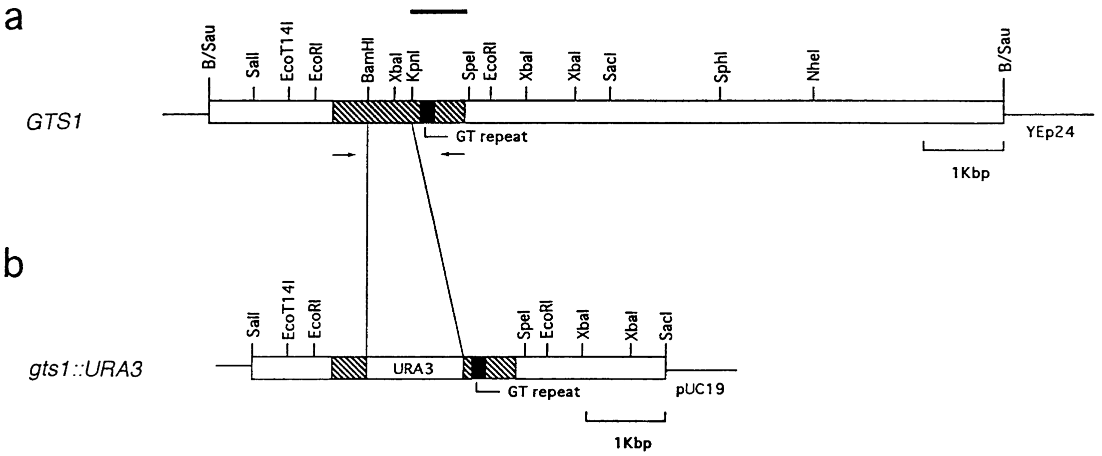

FIG. 1. (a) Physical map of the DNA fragment containing a gene with a GT repeat, named *GTS1*. The fragment was cloned from a genomic library from *S. cerevisiae* by using YEp24 as a vector, and the recombinant plasmid was named pPER119. Hatched and dark boxes indicate the positions of the *GTS1* gene and GT repeat, respectively. The thick line at the top indicates the *Kpn*I-*Spe*I fragment (634 bp) used for Southern and Northern blotting (Fig. 4a and b), and arrows indicate the regions corresponding to synthetic primers for RT-PCR (Fig. 4c). B/Sau, *Bam*HI-*Sau*3AI junction. (b) Physical map of the pPER119-derived DNA fragment with a *GTS1* gene disrupted with the *ura3* gene (*gts1*::*ura3*).

Cells were either cultured in YPAD (rich) medium (2% polypeptone, 1% yeast extract, 40 μg of adenine sulfate per ml, and 2% glucose) or in a synthetic medium consisting of 0.67% yeast nitrogen base without amino acids, 2% glucose, and 20 μg each of histidine, tryptophan, and adenine per ml. Uracil was added at 20 μg/ml when required.

Isolation of the GT repeat-containing gene (*GTS1*). A genomic library constructed in plasmid YEp24 with DNA fragments from *S. cerevisiae* DBY 939 (*suc2-215*^{am}) obtained by partial digestion with *Sau*3AI was screened with ^{32}P-labeled oligonucleotides of the form 5′-d(CCNGTNCCNGTNCC NGTNCC)-3′, which are complementary to the sense strand of DNA encoding three GT repeats (N represents A, G, C, or T). Three positive clones were isolated, and all of them contained mutually overlapping DNA regions. The one with the longest insert (10.1 kbp), named pPER119, was used throughout this study (Fig. 1a). The nucleotide sequence of the 2.4-kbp *Sal*I-*Spe*I fragment was determined by the method of Sanger et al. (41).

Production of transformants. To produce a *GTS1* gene-disrupted transformant, the *GTS1* gene was disrupted with the *URA3* gene according to the method of Rothstein (40). A 4.5-kbp *Sal*I-*Sac*I fragment containing *GTS1* was dissected from pPER119 and inserted into pUC19 in the multicloning site, yielding a recombinant plasmid, pUCGTS. A 442-bp *Bam*HI-*Kpn*I fragment of pUCGTS was then replaced with a *Bam*HI-*Kpn*I fragment containing *URA3*, generating the recombinant plasmid pUCΔGTS1 (*gts1*::*URA3*) (Fig. 1b). A 1,994-bp *Eco*RI-*Eco*RI fragment containing *gts1*::*URA3* was used to transform *S. cerevisiae* IFO10151 or IFO10094 with a *cdc4* mutation by the lithium acetate procedure. Several Ura$^+$ colonies were obtained, and one of the transformants, designated TMΔgts1, was further studied. A high-copy-number transformant of the gene *GTS1*, named TMpGTS1, was obtained by transformation of yeast IFO10151 with the multicopy plasmid YEp24 containing a *Sal*I-*Sac*I fragment from pPER119.

To obtain a Ura$^+$ wild-type cell as a control, IFO10151 was transformed with YEp24 and named WTp24. To rescue the *GTS1*-inactivated transformant, the mitotically stable vector pRS414 containing a centromere sequence (CEN6) (44) was inserted with an *Sph*I-*Sac*I fragment from pPER119 at the multicloning site and the resulting recombinant plasmid (pCT119) was transfected into TMΔgts1, yielding transformant TMΔcGTS1.

Southern and Northern blots. For Southern blots, 10 μg of DNAs prepared from the wild-type and transformed Ura$^+$ strains was digested with either *Hin*dIII, *Pst*I, *Xba*I, or *Eco*RI and hybridized with the ^{32}P-labeled *Kpn*I-*Spe*I fragment from pPER119 (Fig. 1a). For Northern blot analysis of RNA products of *GTS1*, yeast total RNA was extracted with guanidine isothiocyanate (9). Total cellular RNA (20 μg) was separated in a 1.2% agarose gel, blotted onto a nylon membrane, and hybridized with the ^{32}P-labeled oligonucleotides used to screen the gene. When necessary, RNA samples were digested with 2 U of RQ-1 RNase-free DNase (Promega Corp., Madison, Wis.) at 37°C for 30 min in a reaction mixture containing 40 mM Tris-HCl buffer (pH 8), 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$ prior to gel electrophoresis.

Detection of the *GTS1* transcript by PCR. The *GTS1* mRNA was detected by reverse transcription PCR (RT-PCR) as described elsewhere (25). Briefly, 1 μg of total RNA digested with RNase-free DNase was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Grand Island, N.Y.) and random primers (hexadeoxyoligonucleotides) and then amplified with *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) by using N-terminal [5′-d(TAGTCACATATGAGGTTTAG GAGTTCTTCC)-3′] and C-terminal [5′-d(AGTGTAAGCT TATTACTTAGTGTCACTCTA)-3′] synthetic oligonucleotides as primers (Fig. 1a).

Preparation of anti-Gts1 antibody. A *Hin*cII-*Eco*RI fragment from *GTS1* was inserted into pUC119 which was dissected at the *Hin*cII and *Eco*RI site, and then an *Sph*I-*Hin*cII fragment was deleted from the recombinant plasmid to ligate the ORFs of *lacZ* (at nucleotide position +24) and *GTS1* (at +39) in frame to produce pLacZ-GTS1, as shown in Fig. 5a. The construct pLacZ-GTS1 was transfected into *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.), and recombinant Gts1 protein was synthesized in the form of inclusion bodies. These were precipitated from cell lysates by centrifugation at 13,500 × *g* for 5 min at 4°C and by six washes with 0.5% Triton

X-100 containing 25 mM Tris-HCl (pH 8.0), 30 mM NaCl, and 10 mM EDTA (30). The precipitate was then washed eight times with 1 to 8 M urea in 25 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (42) and then dissolved in GTC (5.5 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.1 M β-mercaptoethanol). During dialysis against phosphate-buffered saline, the solution became turbid, and the resultant suspension was centrifuged at 21,000 × *g* for 30 min at 4°C. The precipitate contained a pure protein with a molecular mass of 45 kDa, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (27). This protein matched the molecular mass of the LacZ-Gts1 recombinant protein, while the supernatant contained contaminating proteins. Antiserum was raised against the 45-kDa protein in mice injected four times at 2-week intervals with 100 μg of protein emulsified with complete adjuvant.

Western blots and enzymatic deglycosylation. To prepare protein extracts, cells were harvested, washed once with ice-cold distilled water, resuspended in 50 mM Tris-HCl (pH 6.7)–50 mM NaCl–10 mM β-mercaptoethanol–10% glycerol–protease inhibitors (5 mM EDTA, 10 mM benzamidine HCl, 10 mM *N*-ethylmaleimide, 10 mM aminocaproic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM pepstatin A), and lysed by vortexing in the presence of acid-washed beads. Supernatants were clarified by centrifugation in a microcentrifuge at 15,000 × *g* for 15 min at 4°C. Protein estimation was performed by the Bio-Rad protein assay, and 10 μg of protein was applied to SDS-PAGE. Western blot (immunoblot) analysis was performed essentially according to the method of Towbin et al. (49), and blots were stained by the ECL detection system (Amersham, Amersham, United Kingdom). To investigate the glycosylation state of the Gts1 protein, total protein (10 μg) was digested with endo-β-*N*-acetylglucosaminidase H (8), heparitinase (36), or chondroitinase ABC (36) prior to SDS-PAGE as described previously. Concanavalin A-Sepharose precipitation of total protein was carried out as described by Sweet and Pelham (47).

Determination of growth curves and timing of bud emergence. Cells at a concentration of 10^6/ml were cultured in the synthetic medium at 30°C, and growth curves were determined by measuring the A_{550}. To determine the timing of budding, the cells cultured to the stationary phase in the synthetic medium at 30°C were diluted four times with fresh synthetic medium and cultured at 30°C. At the times indicated, cell samples were removed and the percentage of budded cells was measured under a microscope with a Nomarski lens (BX50; Olympus, Tokyo, Japan) after a brief sonication (22). Dark dead cells were not counted.

Cell cycle synchronization and determination of cell cycle parameters. The cell cycle parameters were determined with virgin cells after synchronization with α-factor. Yeasts were grown at 30°C for 4 days in YPAD medium, and virgin cells were collected on a sucrose density gradient (15). The cells were synchronized with 10 μM α-factor for 2.5 h at 30°C. After the cells were washed five times with 10 volumes of YPAD medium at 4°C, they were suspended in the same medium at a concentration of 10^7/ml and incubated at 30°C. At the times indicated, cell samples were removed and the percentage of budded cells was measured (22). The cell cycle parameters were estimated as explained in the legend to Fig. 7.

Determination of cell size. The cell volumes were determined by measuring the diameters of cells in the log phase. Cells were grown in YPAD medium at 30°C until the A_{600} reached 0.55 and were then transferred to 0°C. After a brief sonication, the cells were appropriately diluted with YPAD medium and photographs were taken under a microscope. The volumes of single cells and mother cells having buds were calculated by measuring the diameters of cells enlarged on photographic papers, assuming that the cells were spherical. The size distribution was determined by flow cytometry using cells in the stationary phase. Cells were cultured at 30°C for 3 days in YPAD medium and examined by flow cytometry (EPICS Profile; Coulter Corp., Hialeah, Fla.) without fixation.

RESULTS

Isolation of a gene with a GT repeat from the yeast *S. cerevisiae*. A genomic library constructed with *Sau*3AI DNA fragments from the yeast *S. cerevisiae* was screened with synthetic oligonucleotides encoding three Gly-Thr repeats as probes. Of three positive clones containing mutually overlapping physical maps, that with the longest insert (10.1 kbp, named pPER119; Fig. 1a) was used throughout this study. As the Gly-Thr/Ser repeat (GT repeat) site was located on the *Kpn*I-*Spe*I fragment (Fig. 1a), the nucleotide sequence of about 2.4 kb around the GT repeat was determined (Fig. 2). There was only one substantial ORF, encoding a protein of 417 amino acid residues containing the GT repeat in frame with a molecular mass of 45,688 (Fig. 2). This ORF does not contain any consensus sequence for an intron. In the 5′ flanking region of the ORF, there were two potential TUF-binding sequences running in different directions with 85% homology to the consensus sequence (50), and an atypical poly(A) addition signal was found in the 3′ flanking region (Fig. 2). The yeast TUF factor is known as a general DNA-binding factor interacting with specific sequences found upstream of a majority of genes encoding components of the translational machinery (like ribosomal proteins) and of many other genes (50). The size of RNA product should therefore be about 1.8 kb. The repeat site was located in the carboxyl-terminal region (nucleotide positions 332 to 358) containing 11 Gly-Ser/Thr dimers encoded by GGCA/TCA with two interruptions. Screening the NBRF and SWISSPROT databases with the sequence deduced from the nucleotide sequence of the *GTS1* gene revealed that a carboxyl-terminal portion of 156 amino acid residues (positions 222 to 378) containing the GT repeat had substantial homology with a portion (positions 593 to 747) of the *per* gene product from *D. melanogaster* (1) (22.6% identity and 61.9% similarity [Fig. 3]). Although the GT repeat region showed particularly high homology, the rest of the homologous region (positions 222 to 332) still had 59% similarity whereas the identity was low (14%).

The gene transcript. Southern blots of DNA digests with either *Hin*dIII, *Pst*I, *Xba*I, or *Eco*RI revealed that yeast possesses a single copy of the gene per haploid (Fig. 4a, for *Eco*RI and *Xba*I digests). The gene transcript of about 1.8 kb, which matches the size predicted from the nucleotide sequence analysis, was detected by Northern blotting (Fig. 4b). The level of the gene transcript was considered to be markedly low and fragile, since an exposure of as long as 7 days was required to detect a very faint signal, even when freshly labeled probes were used, and the 1.8-kb band was usually accompanied by degraded materials. Thus, the presence of the product was confirmed by RT-PCR, which showed one discrete band of the size corresponding to that estimated from the nucleotide sequence of the gene (Fig. 4c). Thus, the gene is considered to be functional and is named *GTS1* (the first gene containing GT dimers from budding yeast). Although the *GTS1* product was the only GT-containing protein isolated by this screening, there is no denying that there are more GT proteins in *S. cerevisiae*.

To detect the protein product of the *GTS1* gene in *S.*

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   1  GTCGACAAATACACCGGTGGCACGGAGGCTGAATGTTGGTCATTTTGAGAGCCGGTATT    59
  60  TACCACAACTGTATCTAATCTCTTGATCGGCATCTTTTTGCTTCTATGCTTTTGTTGTTT   119
 120  GCTTGATATTTTTTGTTTTTAATGAACTGTAAGCACACAGAGCTATGGGATGTGTGGCAA   179
 180  TGACTGTATATATATGTATATATATATATATATATATATATATTTATTTATTTATTTA   239
 240  TTTAAAATATAGCTACGTATGCGCCAGTTGTTTTTATCAGCTTTGCTTCTGGCCACATTT   299
 300  CAACCAGGTCTCAGAGGATATATGGGCCAGAAGGCCCCTTTACACACTTTTGAGATGCGA   359
 360  TGAGTCTTCCAAGGTAGGAGCATATGCCTCCAACAGACTTCGGCCCGAAGCATGCTGTGC   419
 420  TCACGGGAATTATATTGTGTGAAGCACATGGCACACTTTCCAAAAAAGAGTCAGAATGAG   479
 480  TCAGCCGGATAACCAAACGCGGACTGTGGCGCCACGGAAAACAGTGTGGCAAATTTATGG   539
 540  GCGGCTAATCTGCAAGCTCTATGCCTGGAAATGTAGATGTAATAATCAAGACACTTTTGT   599
 600  TCTTTCGTGTATAGTGCATGAAGGTTAAACCTCCGTAAATTCCTTGAACTCTTCTGGAAT   659
 660  TCCAAATTCTTTTCTTATCTGTTCTGGCTTCAAAAAGAATTTCTTGTATAAATAATCAAT   719
 720  GAGAATCTCAATGTTATCAGTAGTTTTCTCCAGATGCACTTTTTTTAGGCGGATCTCTAA   779
 780  ATGCTTCACAAAAGGGTGGAAGAAGAACATTTTCTCTTGATCAATCATTAATATAGATGG   839
 840  AGGCAGCGTTCTGTCAATCACAGATTATAGTTTATTTATACTTAGTCAAAAATGAGGTTT   899
                                                                M  R  F        3
 900  AGGAGTTCTTCCCATAGCTTAAAACATGTTGACAGAGAATTAAAGGAGTTGATTAATTCG   959
   4  R  S  S  S  H  S  L  K  H  V  D  R  E  L  K  E  L  I  N  S      23
 960  TCCGAGAATGCCAATAAATGCGGTGAATGTGGTAATTTCTACCCAACTTGGTGCTCAGTT  1019
  24  S  E  N  A  N  K  C  G  E  C  G  N  F  Y  P  T  W  C  S  V      43
1020  AATTTGGGGGTTTTCCTTTGTGGTAGGTGTGCCTCTGTTCATAGAAAAGTTTTCGGTAGC  1079
  44  N  L  G  V  F  L  C  G  R  C  A  S  V  H  R  K  V  F  G  S      63
1080  AGAGACGACGATGCCTTTTCTAACGTTAAATCACTATCAATGGATAGATGGACAAGAGAG  1139
  64  R  D  D  D  A  F  S  N  V  K  S  L  S  M  D  R  W  T  R  E      83
1140  GATATCGATGAATTAGTGAGTCTTGGAGGCAATAAGGGGAATGCTCGGTTCTGGAATCCT  1199
  84  D  I  D  E  L  V  S  L  G  G  N  K  G  N  A  R  F  W  N  P     103
1200  AAAAATGTCCCTTTTCCTTTTGATGGAGATGATGACAAAGCCATCGTGGAGCATTACATT  1259
 104  K  N  V  P  F  P  F  D  G  D  D  D  K  A  I  V  E  H  Y  I     123
1260  AGAGACAAGTATATTTTGGGTAAATTCAGGTATGATGAAATAAAGCCTGAAGACTTTGGA  1319
 124  R  D  K  Y  I  L  G  K  F  R  Y  D  E  I  K  P  E  D  F  G     143
1320  TCCAGAATGGATGATTTTGATGGGGAATCGGACAGGTTTGATGAAAGAAATAGAAGTAGG  1379
 144  S  R  M  D  D  F  D  G  E  S  D  R  F  D  E  R  N  R  S  R     163
1380  AGCAGGAGCAGATCTCATTCTTTCTATAAAGGGGGCCATAATAGGTCTGACTACGGCGGT  1439
 164  S  R  S  R  S  H  S  F  Y  K  G  G  H  N  R  S  D  Y  G  G     183
1440  TCCAGGGACTCATTCCAAAGCAGTGGAAGCAGATATTCTAGGCAACTGGCAGAACTCAAG  1499
 184  S  R  D  S  F  Q  S  S  G  S  R  Y  S  R  Q  L  A  E  L  K     203
1500  GACATGGGTTTCGGTGATACAAACAAAAATTTAGATGCATTATCGTCCGCTCACGGAAAT  1559
 204  D  M  G  F  G  D  T  N  K  N  L  D  A  L  S  S  A  H  G  N     223
1560  ATCAATAGAGCAATCGATTATCTAGAAAAAAGTTCAAGTTCAAGAAATAGTGTATCGGCA  1619
 224  I  N  R  A  I  D  Y  L  E  K  S  S  S  S  R  N  S  V  S  A     243
1620  GCAGCGACAACATCAACTCCGCCCCTGCCCAGGAGACGTGCGACAACAAGTGGCCCACAG  1679
 244  A  A  T  T  S  T  P  P  L  P  R  R  R  A  T  T  S  G  P  Q     263
1680  CCAGCTATTTTTGATGGTACAAATGTAATCACGCCAGATTTTACTTCAAATTCAGCATCT  1739
 264  P  A  I  F  D  G  T  N  V  I  T  P  D  F  T  S  N  S  A  S     283
1740  TTTGTACAAGCAAAGCCAGCAGTTTTCGATGGTACCCTTCAACAGTATTACGATCCTGCT  1799
 284  F  V  Q  A  K  P  A  V  F  D  G  T  L  Q  Q  Y  Y  D  P  A     303
1800  ACCGGAATGATATATGTAGATCAGCAGCAATACGCCATGGCTATGCAACAGCAGCAACAG  1859
 304  T  G  M  I  Y  V  D  Q  Q  Q  Y  A  M  A  M  Q  Q  Q  Q  Q     323
1860  CAACAGCAACAGCTTGCTGTTCGCACAGGCTCAGGCTCAGGCACAGGCACAGGCACAGGC  1919
 324  Q  Q  Q  Q  L  A  V  R  T  G  S  G  S  G  T  G  T  G  T  G     343
1920  TCAAGTTCAGGCTCAGGCTCAGGCTCAGGCCCAGGCTCAGGCTCAAGCACAGCAGATCCA  1979
 344  S  S  S  G  S  G  S  G  S  G  P  G  S  G  S  S  T  A  D  P     363
1980  GATGCAACAGCTTCAGATGCAGCAACAGCAACAGGCGCCATTATCTTTTCAGCAAATGTC  2039
 364  D  A  T  A  S  D  A  A  T  A  T  G  A  I  I  F  S  A  N  V     383
2040  GCAAGGAGGAAACCTGCCACAAGGTTATTTCTACACACAATAAAAATAAGATACATTGCC  2099
 384  A  R  R  K  P  A  T  R  L  F  L  H  T  I  K  I  R  Y  I  A     403
2100  GCTGCTCCGCATTACATATCATATAGAGTGACACTAAGTAATTAAATGACACTAAGTAAC  2159
 404  A  A  P  H  Y  I  S  Y  R  V  T  L  S  N ***
2160  GAAAGATATCTGTAAACAACGACTATATACCAGAATTAGTCATATTCTCTATTGTATTCA  2219
2220  CCCTACACAGCCGCATTACACAGCTGCTCCGGACGTTTTACGCTGCACATCAATGACTAA  2279
2280  TACCACTGTATTTGATCCACACTTAGCGCAAAAGTCACCTGGGTGGCGTTTTCCGCTCGC  2339
2340  GCTCCGCCGGTGAGAGCGAGAAAAAAAAAAAGGGATTGGGCGGTAATGTAAGGAAAACCCA  2399
2400  CTAGA
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FIG. 2. The nucleotide sequence of the 2.4-kbp *Sal*I-*Spe*I fragment and the deduced amino acid sequence of the largest ORF. The GT repeat site is underlined with a heavy line; the predicted TUF factor-binding sites (nucleotide positions 613 and 629) and poly(A) addition signal (position 2194) are underlined with light lines.

cerevisiae, a polyclonal antibody was raised against a recombinant LacZ-Gts1 protein overproduced in *E. coli* by using a recombinant plasmid (Fig. 5a). Western blots using the antibody detected a positive protein of about 45 kDa, which is close to the size predicted from the nucleotide sequence (Fig. 5b), suggesting that the Gts1 protein was expressed in growing yeast cells. Digestions of the protein extracts with enzymes that remove carbohydrate side chains (endo-β-*N*-acetylglucosaminidase H, heparitinase, and chondroitinase ABC) have no effect on the mobility of the Gts1 protein, as determined by PAGE. Furthermore, the protein did not bind to the lectin concanavalin A, unlike most yeast glycoproteins, as determined by concanavalin A-Sepharose precipitation (data not shown). Thus, the Gts1 protein appeared unglycosylated.

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             190       200       210       220       230       240
GTS1  YGGSRDSFQSSGSRYSRQLAELKDMGFGDTNKNLDALSSAHGNINRAIDYLEKSSSSRNS
                                              >:.:..  :: ...::. ..
PER   RCQALASFMETLMDEVSRADLKLELPHENELTVSERDSVMLGEISPHHDYYDSKSSTETP
            560       570       580       590       600       610

             250       260       270       280       290       300
      VSAAATTSTPPLPRRRATTSGPQPAIFDGTNVITPDFTSNSASFVQAKPAVFDGTLQQYY
       : .. . .  : :  .... ..:: .: ... .:.    ... : . .. .. . .
      PSYNQLNYNENLLRFFNSKPVTAPAELDPPKTEPPE---PRGTCVSGASGPMSPVHEGSG
            620       630       640          650       660

             310       320       330       340       350       360
      DPATGMIYVDQQQYAMAMQQQQQQQQQLAVRTGSGSGTGTGTGSSSGSGSGSGPGSGSST
      .....  .... .  :.   .   .   .. ::.:.:::::::...:.:.:.:.:.:..:
      GSGSSGNFTTASNIHMSSVTNTSIAGTGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
     670       680       690       700       710       720

            370       380       390       400       410
      ADPDATASDAATATGAIIFSANVARRKPATRLFLHTIKIRYIAAAPHYISYRVTLSN
      .. ..:.....:....<
      GTGTGTGTGNGTNSGTGTGTASSSKGGSAAIPPVTLTESLLNKHNDEMEKFMLKKHR
     730       740       750       760       770       780
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FIG. 3. Homology of carboxyl-terminal segments of putative *GTS1* and *per* (*D. melanogaster*) gene products showing 22.6% identity in a defined region (> to <). Colons and dots indicate identical and similar amino acid residues, respectively.

Production of transformants. To study the function of the gene in *S. cerevisiae*, we constructed two kinds of transformants of IFO10151 and investigated their phenotypes. One is the gene-disrupted transformant, named TMΔgts1, whose *GTS1* gene was inactivated by transformation with a disrupted *GTS1* gene (*gts1*::*URA3*) (Fig. 1b). The other was a high-copy-number transformant, named TMpGTS1, which was transformed with a recombinant plasmid of YEp24 containing a *Sal*I-*Sac*I fragment from pPER119. The integration of the disrupted gene *gts1*::*URA3* in a homologous region was confirmed by Southern blotting (Fig. 4a). The *Eco*RI and *Xba*I fragments from transformant TMΔgts1 were longer than those from the wild-type cell because of insertion of the *URA3* gene and elimination of an *Xba*I site, respectively, in agreement with the physical maps (Fig. 1). Northern blots (Fig. 4b) showed that the high-copy-number transformant TMpGTS1 contained much more of the 1.8-kb RNA product than did the wild type (Fig. 4b) and that it was undetectable in the gene-disrupted transformant TMΔgts1 (Fig. 4b). The absence of the transcript in TMΔgts1 was confirmed by RT-PCR (Fig. 4c). Furthermore, Western blots showed that transformant TMpGTS1 contained about 20 times more protein than did the wild-type cell, whereas transformant TMΔgts1 contained none (Fig. 5b). As transformant TMΔgts1 grew in the absence of the Gts1 protein, the protein was shown not to be essential for cell viability.

Effect of the gene on cell growth. To examine whether the gene affects cellular growth rate, growth curves for the wild type and the two transformants during the culture in the synthetic medium at 30°C were compared by monitoring the turbidity at 550 nm (Fig. 6a). Although their growth rates were similar during the exponential growth phase, the lag phase was reproducibly the shortest for transformant TMΔgts1 and longest for the multicopy transformant TMpGTS1, although the difference between the transformants and the wild-type cells was small. To confirm the difference in the duration of the lag phase, the timing of the first bud formation was determined after the addition of fresh medium (Fig. 6b). As expected, the timing of the first budding was shortest for TMΔgts1 and longest for TMpGTS1, although the timing was generally shorter in this experiment than that in the experiment for Fig. 6a, probably because the initial cell concentration was higher. Thus, the timing of bud emergence after the transfer to the fresh medium was affected by the *GTS1* gene and roughly

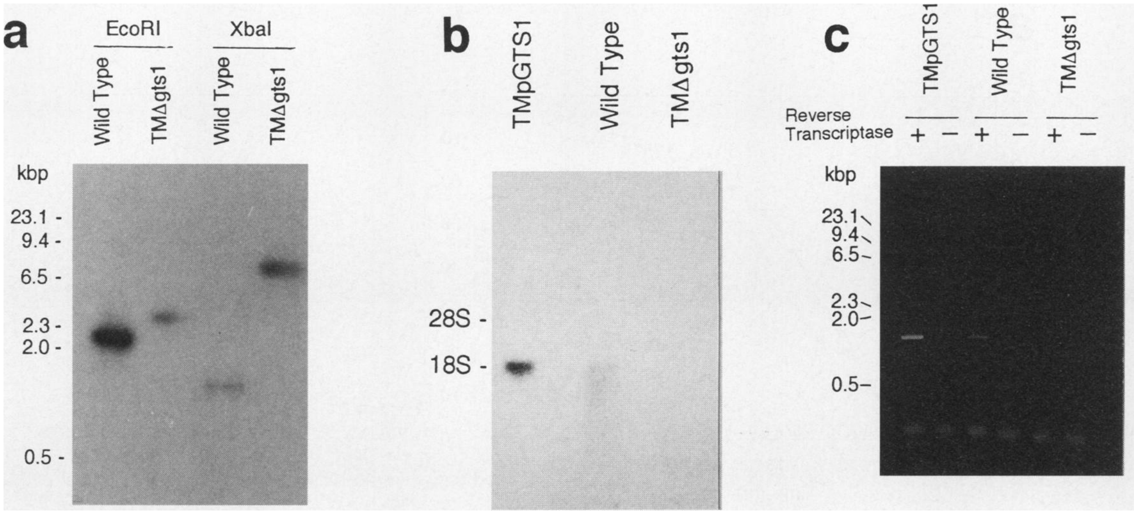

FIG. 4. (a) Southern blot of the *GTS1* gene in DNAs from the wild-type strain (IFO10151) and transformant TMΔgts1, which was produced by transformation with the 1,994-bp *Eco*RI-*Eco*RI fragment containing *gts1::URA3*. DNAs (10 μg) were digested with *Eco*RI or *Xba*I and hybridized with the ^{32}P-labeled *Kpn*I-*Spe*I fragment from pPER119 as marked in Fig. 1a. The lengths of the DNA fragments were determined with *Hin*dIII fragments of λ phage DNA as the standard. (b) Northern blots of RNA products of the *GTS1* gene. Total cellular RNAs from TMpGTS1 (the high-copy-number transformant with pPER119), the wild-type cell, and TMΔgts1 (10 μg) were separated in a 1.2% agarose gel, blotted onto a nylon membrane, and hybridized with the ^{32}P-labeled *Kpn*I-*Spe*I fragment from pPER119. (c) Detection of *GTS1* mRNA by RT-PCR (29). One microgram of total RNA from the wild-type cell, TMΔgts1, and TMpGTS1 was reverse transcribed into cDNA with reverse transcriptase (lanes +) by using random primers (hexadeoxyoligonucleotides) and then amplified with *Taq* DNA polymerase by using N- and C-terminal synthetic oligonucleotides (as marked in Fig. 1a) as primers. Control experiments without reverse transcriptase (lanes −) show the absence of DNA contamination in RNA samples.

dependent on the level of the Gts1 protein. That is, the more protein was present, the longer the period of the lag phase of culture was.

Ura$^+$ wild-type WTp24 cells produced by transformation with YEp24 followed essentially the same time course of budding as that of the Ura$^-$ wild-type cells, ruling out the effect of uracil on the growth rate (data not shown). Furthermore, TMΔcGTS1, which was produced by transformation of the gene-disrupted transformant TMΔgts1 with a centromere plasmid harboring the *GTS1* gene (pCT119), restored the timing of budding to that of the wild-type cells (Fig. 6b), suggesting that the timing of budding was affected by the *GTS1* gene.

The low percentage of budding cells at peaks in Fig. 6b,

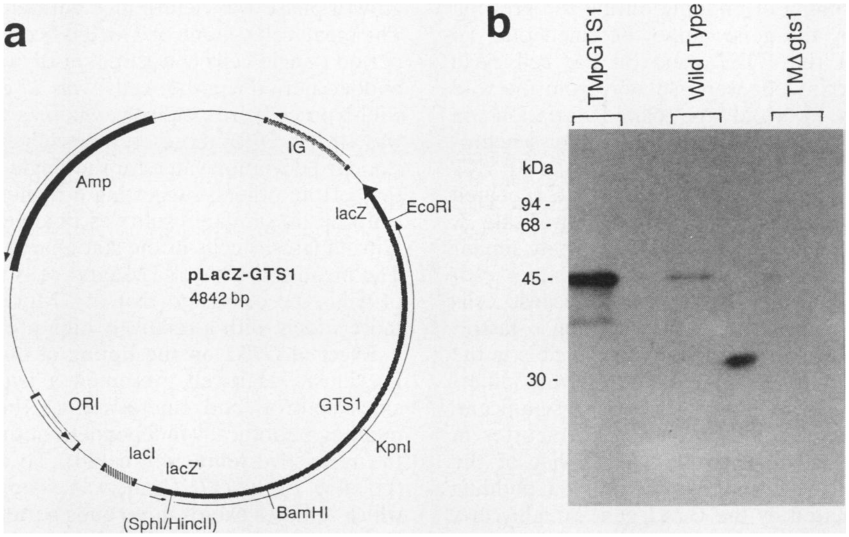

FIG. 5. Western blots using antisera raised against LacZ-Gts1 recombinant protein. (a) The structure of the recombinant plasmid pLacZ-GTS1. Plasmid pUC119 was inserted with the *Hin*cII-*Eco*RI fragment from *GTS1*, and the ORFs of *lacZ* (at nucleotide position +24) and *GTS1* (at +39) were ligated in frame. (b) Total proteins from TMpGTS1 (the high-copy-number transformant with pPER119), the wild-type cell, and TMΔgts1 (10 μg each) were separated on a polyacrylamide gel and electrophoretically blotted. The Gts1 protein was detected by the ECL method with antiserum raised against the LacZ-Gts1 recombinant protein purified from *E. coli* and overexpressed, depending on recombinant plasmid pLacZ-GTS1.

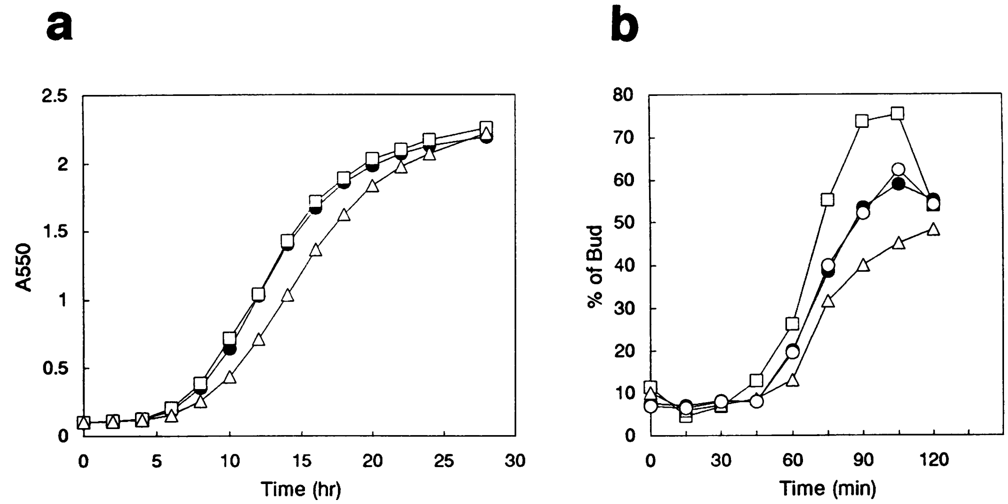

FIG. 6. (a) Growth curves of the wild-type strain (●) and transformants TMΔgts1 (□) and TMpGTS1 (△). Cells at a concentration of 10^6/ml were cultured in the synthetic medium at 30°C, and growth curves were determined by measuring the A_{550}. (b) Time course of budding of the wild-type strain (●) and transformants TMΔgts1 (□), TMpGTS1 (△), and TMΔcGTS1 (○). TMΔcGTS1 is TMΔgts1 cells transformed with a centromere plasmid inserted with the *GTS1* gene. Cells cultured to the stationary phase in synthetic medium at 30°C were diluted four times with fresh medium and transferred to 30°C at time zero. At the times indicated, cell samples were removed and the percentage of budded cells was determined by microscopy after a brief sonication.

especially that of the multicopy transformant TMpGTS1, was not due to an increase of dead cells, because only live cells were counted by Nomarski imaging. It is probably because the cell populations are of various ages having different growing activities, and so cell-to-cell variations in the timing of budding spread as the average timing of the population was delayed. However, after taking it into account, the timing of budding of TMpGTS1 was considered to be extremely varied compared with that of the other cells. This may be due to variations in the copy number of the plasmid harboring the *GTS1* gene.

Effect of the gene on timing of bud emergence during the cell cycle. Although both transformants showed growth rates similar to that of the wild-type, according to the turbidity (Fig. 6a), the possibility that the timing of budding during the growing phase is also affected by the gene cannot be ruled out. To determine the effect of the *GTS1* gene on the cell cycle parameters precisely, virgin cells were isolated from the wild type and transformants to avoid age-related variations in growth activity, and their growth was examined after synchronization with α-factor, which stops the cell cycle at the START point (21, 23) (Fig. 7). Identical experiments were repeated three times, and the results were summarized in Table 1. Although overall profiles of the time courses were quite similar among the experiments, the standard deviation values estimated were somewhat large. This is probably because cells were sensitive to synchronization conditions using α-factor. Anyway, the most significant changes were reproducible in the unbudded periods of mother (parent) cells, whereas differences among them in the budded period were not significant. The unbudded period of mother cells was 19% shorter in TMΔgts1 and 18% longer in TMpGTS1 than that of the wild-type cells. This result suggested that the timing of budding of mother cells is modulated by the *GTS1* gene roughly in a dose-dependent manner. It should be noted that transformant TMΔcGTS1 showed essentially the same time course as that of the wild type (Fig. 7), indicating again that the *GTS1* gene affected the timing of budding in TMΔgts1.

The result also showed that the unbudded periods of the daughter (virgin) cells from the START point were changed similarly to those of mother cells (Table 1). Furthermore, isolated virgin cells grown in a rich medium without α-factor followed essentially the same time course of bud emergence as those shown in Fig. 6b, in which the cell populations are of various ages. The length of the unbudding period after the first bud emergence was shortened for TMΔgts1 and lengthened for TMpGTS1 by about 20% compared with that for wild-type cells (data not shown). These results suggested that the timing of budding of daughter cells is also affected by the gene.

Effect of *GTS1* on cell size. As the *GTS1* gene affects the duration of the unbudded phase of the cell cycle while little affecting the growth rate, we presumed that cell sizes are affected. The size distribution of cells in the exponential growth phase was determined without cell fixation (Table 2). The mean cell volume of TMΔgts1 cells in either the unbudded period (single cells consisting mostly of daughter cells) or the budded period (mother cells) was 27 or 31% less than that of wild-type cells, respectively, whereas that of TMpGTS1 cells was 48 or 39% more, respectively. It is notable that the standard deviation value for the single TMpGTS1 cells is twice that of the others, suggesting a higher degree of variation in cell size. A similar result was obtained by flow cytometry of unfixed (fresh) cells in the stationary phase (data not shown). The mean cell sizes of TMΔgts1 cells were smaller than those of wild-type cells, and that of TMpGTS1 cells is distributed more widely with a relatively high proportion of large cells.

Effect of *GTS1* on the timing of budding of *cdc4* mutants. Previously, Hartwell presented a hypothesis that a cellular clock controls bud emergence, as the *cdc4* mutant initiates budding periodically independent of the DNA division cycle at the restrictive temperature (20). To address this hypothesis, the effect of the *GTS1* gene was examined with a *cdc4* mutant which initiates two or three buds periodically in the absence of DNA replication and cell separation at the restrictive temperature (20). A gene-disrupted transformant (TMΔgts1/cdc4) and an overexpressed transformant (TMpGTS1/cdc4) were constructed from the *cdc4* mutant, and the time course of budding was determined after transfer to the restrictive temperature (Fig. 8). Although determination of the interval

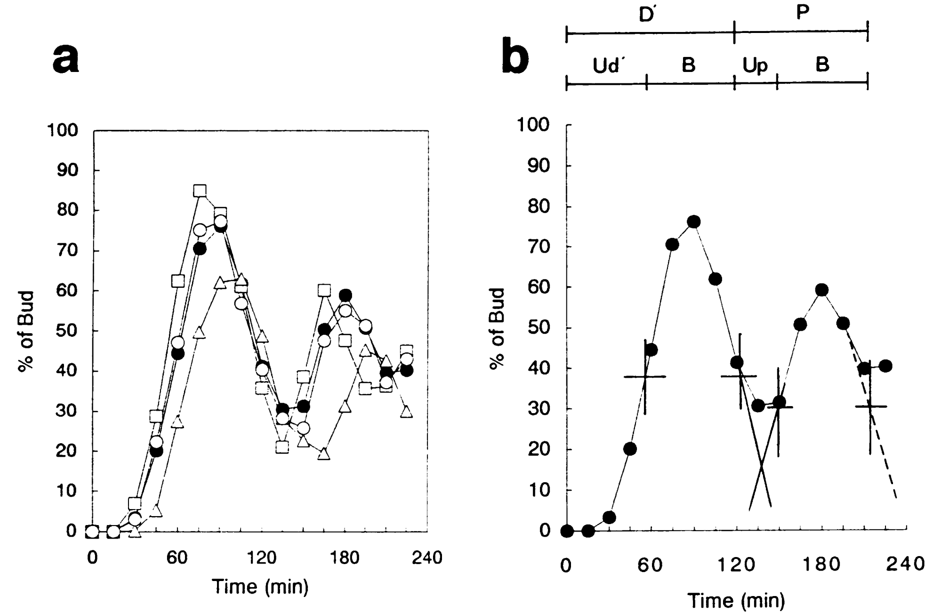

FIG. 7. (a) Cell cycle of virgin cells from the wild-type strain (IFO10151) (●) and transformants TMΔgts1 (□), TMpGTS1 (△), and TMΔcGTS1 (○) after synchronization with α-factor. Virgin cells collected on a sucrose density gradient were synchronized with 10 μM α-factor for 2.5 h at 30°C. After extensive washing with fresh YPAD medium at 4°C, they were suspended at a concentration of 10^7 cells per ml of YPAD medium and transferred to 30°C at time zero. At the times indicated, cell samples were removed and the percentage of budded cells was measured. (b) The cell cycle parameters were roughly estimated and are summarized in Table 1. Abbreviations for the cell cycle parameters: D' and P, generation times of daughter (virgin) and parent cells, respectively; Ud' and Up, unbudded periods of daughter and parent cells, respectively; B, budded periods of daughter and parent cells, which are identical (20). Here, Ud' was defined as the period from the time of release from α-factor to the midpoint of the ascending slope of the first peak (the mean time of appearance of the first bud). Thus, it was probably shorter than the normal Ud (15), as the cells were arrested in the late G_1 phase by α-factor. The daughter cells from the first bud (the first peak) initiated budding later than those from the second bud of the parent cells (the second peak), consequently making the second trough shallow.

between the first and second buddings was impossible as the synchronization of the cell cycle was too incomplete, it is apparent that the timing of budding, at least that of the first bud, is shortened in TMΔgts1/cdc4 and delayed in TMpGTS1/cdc4 compared with that in control *cdc4* cells. Thus, it is suggested that the *GTS1* gene product influences the timing of budding independent of the DNA division cycle.

DISCUSSION

Characterization of the *GTS1* gene. In this study, we isolated a gene, named *GTS1*, from the yeast *S. cerevisiae* by using synthetic oligonucleotides encoding three GT repeats as probes which are shared by clock-affecting genes from *D. melanogaster* and *N. crassa* (24, 31, 37). A positive clone of 10.1 kbp in length contained an ORF encoding a protein of 417 amino acid residues without any consensus sequence for an intron (Fig. 2). The gene was considered functional because the RNA and protein products detected by Northern and Western blotting, respectively, had molecular sizes matching those predicted by the nucleotide sequence. Furthermore, a PCR product of a size identical to that predicted from the nucleotide sequence was detected, although the complete cDNA has not been cloned yet.

Structural significance of the predicted *GTS1* product. The *GTS1* product predicted from the nucleotide sequence is much smaller than the *per* and *frq* proteins, which have 1,218 (type A,

TABLE 1. Summary of cell cycle parameters of the wild type (IFO10151) and transformants TMΔgts1 and TMpGTS1

Strain	Length (min ± SD) of cell cycle parameter[a]			
	Ud'	B	Up	P
Wild type	57.0 ± 4.3	51.7 ± 6.8	32.8 ± 3.8	85.3 ± 5.9
TMΔgts1	48.0 ± 4.2	53.3 ± 6.0	26.3 ± 4.1	81.3 ± 10.9
TMpGTS1	64.3 ± 6.3	52.7 ± 5.8	37.7 ± 5.1	92.0 ± 12.3

[a] Cell cycle parameters are estimated from the results of three independent experiments as described in the legend to Fig. 7, and the abbreviations are defined there. Briefly, virgin cells were synchronized with 10 μM α-factor. After extensive washing with fresh YPAD medium at 4°C, they were suspended in YPAD medium (10^7 cells per ml) and transferred to 30°C at time zero. Time courses of budding were determined by measuring the percentage of budded cells, and cell cycle parameters were estimated.

TABLE 2. Distribution of cell volume of the wild type (IFO10151) and transformants TMΔgts1 and TMpGTS1

Strain	Results[a] (mean ± SD) for:			
	Single cells		Mother cells	
	Diam (μm)	Vol (μm^3)	Diam (μm)	Vol (μm^3)
IFO10151	4.09 ± 0.35	35.8 ± 3.1	5.12 ± 0.38	70.2 ± 5.2
TMΔgts1	3.70 ± 0.30	26.5 ± 3.4	4.52 ± 0.40	48.3 ± 6.9
TMpGTS1	4.66 ± 0.70	53.0 ± 6.3	5.71 ± 0.39	97.4 ± 6.7

[a] The cell volumes were determined by measuring the diameters of cells in the log phase. Cells were grown in YPAD medium at 30°C until the A_{600} reached 0.55. After a brief sonication at 0°C, the cells were appropriately diluted with YPAD medium, and photographs were taken under a microscope. The volumes of single cells and mother cells having buds were calculated by measuring the diameters of cells enlarged on photographic papers.

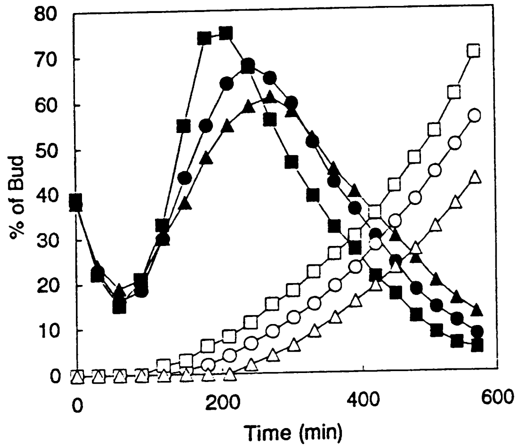

FIG. 8. Time course of budding of the *cdc4* mutant (IFO10094) (● and ○) and transformants TMΔgts1/cdc4 (■ and □) and TMpGTS1/cdc4 (▲ and △) after transfer to the restrictive temperature. Cells were grown overnight at 23°C in YPAD medium and transferred to 37°C at time zero. At the times indicated, samples of cells were removed and the percentages of cells with one (closed symbols) and two (open symbols) buds were measured by microscopy after brief sonication.

the most abundant transcript) and 788 residues, respectively. However, screening the protein databases revealed that a carboxyl-terminal portion of 156 amino acid residues containing the GT repeat site has substantial homology with a portion of the *per* product (Fig. 3). As the *frq* protein reportedly is similar to the *per* protein in the region containing the GT repeat (31), it is likely that *GTS1* is evolutionarily related to *per* and *frq*. The GT/S motif constitutes the attachment site of the glycosaminoglycan chain to the core protein of proteoglycans (5), and the *per* product is attached to a sugar chain which is sensitive to heparitinase (37) and heparinase II (2). However, a polyclonal antibody against the Gts1 protein reacted with a protein of 45 kDa, which approximately matches the size predicted by the nucleotide sequences of the gene, and both assays of enzymatic deglycosylation and concanavalin A-Sepharose binding suggested that the Gts1 protein was unglycosylated. In addition to the GT repeat, the Gts1 protein contains a glutamine-rich tract which is shared among Per-related proteins that are similar to the *per*, *ARNT*, and *SIM* products (32) and some nuclear proteins, such as the transcription regulatory factor HAP2 and homeotic proteins in the fruit fly. However, unlike these proteins, the Gts1 protein does not contain a typical nuclear targeting sequence (N-MIIKRNK DKS-**RKKKKNK**) (38), although it has some strands of basic amino acids. This does not mean, however, that Gts1 is absolutely a cytoplasmic protein, since about 50% of nuclear proteins do not contain the nuclear targeting sequence (38).

Function of the *GTS1* gene in *S. cerevisiae*. The function of the *GTS1* gene was examined by characterizing the phenotypes of gene-disrupted (TMΔgts1) and high-copy-number (TMpGTS1) transformants of the gene. The phenotypes of the transformants were found in the growth curves and the cell cycle parameters with or without α-factor synchronization. The unbudding period of the mother as well as daughter (virgin) cells changed as a function of the gene dosage. In other words, the period of the gene-disrupted transformant TMΔgts1 was shortened and that of the transformant TMpGTS1 was lengthened compared with the period of wild-type cells. Furthermore, the *GTS1* gene influences the timing of budding independent of the cycle consisting of DNA replication, nuclear division, and cytokinesis using the *cdc4* mutation (Fig. 8).

Another significant change in transformants of the *GTS1* gene was in the distribution of cell size. Direct measurements of the cell diameter in the log phase showed that the mean cell volumes of mother and daughter TMΔgts1 cells were about 30% smaller than those of the wild-type, whereas those of TMpGTS1 cells were about 44% larger (Table 2). This is supported by observations from flow cytometry of the cells in the stationary phase. These results suggested that when the *GTS1* gene is inactive, the cell begins to bud earlier than normal, resulting in premature budding and consequently the formation of smaller cells. On the other hand, when the *GTS1* gene is overexpressed, budding is delayed to a somewhat variable extent and abnormally large cells are formed. In turn, this means that the *GTS1* gene primarily affects the timing of budding rather than the growth rate in *S. cerevisiae*. The low ability of TMpGTS1 to control the cell size and the timing of bud emergence may be due to cell-to-cell variations in the copy number of the plasmid harboring the *GTS1* gene.

Previously, Edmunds et al. (14) demonstrated that the yeast *S. cerevisiae* shows a circadian rhythm in cell division activity when the cells are cultured at low temperatures to elongate the generation time. At the same time they reported the incomplete synchronization of cell division and difficulties in obtaining clear-cut rhythmicity in the yeast (14). We could not reproducibly synchronize cell division with a light-and-dark cycle, although we followed their methods and changed the duration of the cycle and intensity of the illumination. Thus, it is premature to refer to the *GTS1* gene as a yeast clock gene. In this respect, *Euglena gracilis* may be more useful, as its cell division is easily synchronized with the light-and-dark cycle (1).

So far, several cell size mutants have been isolated in *S. cerevisiae*. Strains with the nonlethal mutation in *WHI1* (13, 46), which was later identified as a dominant mutation of cyclin 3 (*CLN3*) (33), as well as in the *WHI2* locus, produce abnormally small cells (13). The products of *CDC25* (a modulator of yeast adenylate cyclase) and *RAS2* also participate in cell size modulation via the regulation of cyclic AMP metabolism, which controls the critical size for bud initiation (3, 6, 7, 39). More recently, an *RCS1* locus whose disruption mutant produces cells that are twice the size of cells of the parental strain has been reported (17). At present, it is generally recognized that cyclins and *cdc28* kinase play a major role in the regulation of cell size by controlling the rate of the cell cycle in yeasts and higher organisms (28, 29). Differing from these genes, whose inactivation increases the cell size, inactivation of the *GTS1* gene decreases the cell size. The functional mechanism of the *GTS1* gene should be studied in relation to these genes, especially to those of cyclins.

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