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A yeast gene required for the G_1-to-S transition encodes a protein containing an A-kinase target site and GTPase domain

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A new temperature-sensitive mutant of *Saccharomyces cerevisiae*, *gst1* (G_1-to-*S* transition) was isolated. At non-permissive temperature the mutant cells with large buds accumulated and DNA synthesis was substantially arrested. From the reciprocal experiment of temperature-shift and mating-factor treatment, it was shown that the execution point was post 'START'. This suggested that the mutation affected the G_1-to-S phase transition in the cell cycle. A DNA clone complementing the *gst1-1* mutation was isolated from a yeast gene library, and *gst1* was mapped in chr4R, by Southern blotting of cloned sequence to the individual yeast chromosome DNA by OFAGE system and by genetic analysis. The gene product was tentatively assigned from DNA sequencing analysis, as a protein of mol. wt 76 565 which contained consensus sequences for a target site of cAMP-dependent protein kinase(s) and for GTPase with extensive homology to polypeptide chain elongation factor EF1α.
Key words: cell cycle/DNA synthesis/EF1α homolog/site for A-kinase/yeast *ts* mutant

Introduction

To study the eukaryotic cell cycle, the budding yeast *Saccharomyces cerevisiae* offers a useful model system. Mutations causing stage-specific arrest in the cell cycle are important to clarify what kinds of gene products are involved in those complex processes, and the characterization of these proteins gives us clues to understand how they operate (Hartwell *et al.*, 1973; Pringle and Hartwell, 1981; Reed *et al.*, 1985; Patterson *et al.*, 1986). The initial event in the cell cycle, called 'START', takes place in the late G_1 phase, where the decision to undergo one cell cycle is made only when most of the cellular molecules are ready to be duplicated. Several *cdc* (*c*ell *d*ivision *c*ycle) mutants were isolated which affected this 'START' event (Hartwell, 1973; Hartwell *et al.*, 1974; Reed, 1980; Bedard *et al.*, 1981). Once a cell cycle has passed through 'START', DNA synthesis initiates and the normal sequential events of the cell cycle follow. For the subsequent step, but immediately prior to the DNA synthesis, two *cdc* genes, namely *cdc4* and *cdc7*, have been known to function in this order (Hereford and Hartwell, 1974). After the DNA is duplicated, each chromosome connected via *cis*-acting centromeres to the mitotic apparatus is faithfully distributed to each daughter cell. The yeast is the only organism whose centromere sequences have been cloned and characterized (Clarke and Carbon, 1980; Stinchcomb *et al.*, 1982).

Our initial aim was to isolate temperature-sensitive mutants in which proteins acting on centromeres were defective. For this purpose we took advantage of the fact that the chromosome copy number was rigorously regulated by the number of the centromere, although the mechanism which maintains the copy number of each chromosome to be one per haploid cell is yet unknown. When the centromere sequence is incorporated into multicopy plasmids such as yeast 2μ-plasmid, the copy number of the hybrid plasmid drops as low as one molecule per cell (Stinchcomb *et al.*, 1982). If proteins exist that control the chromosome copy number at the centromere level, we might expect to obtain mutants with thermolabile proteins where the hybrid 2 μ-plasmid with centromere sequence could amplify to a higher copy number at an intermediate temperature. The amplification might be facilitated through the intramolecular inversion system of the 2 μ-plasmid mediated by *FLP*-protein between two inverted repeat sequences (Futcher, 1986; Volkert and Broach, 1986). To detect amplified hybrid plasmids, we connected a defective *LEU2* (Beggs, 1978) to the plasmid as a selective marker which expressed only 5% of the wild-type level (Erhart and Hollenberg, 1983), so that we could get Leu^+ transformants when the copy number increased. Unexpectedly, we obtained a group of mutants in which the copy number of CEN-plasmid did not increase and whose cell cycles were arrested at the stage prior to DNA synthesis at high temperature. In this report, one of the mutants, *gst1*, was analysed. The new gene appeared to be required for the G_1-to-S phase transition and encoded a protein with a target site of cAMP-dependent protein kinase(s) (Cohen, 1985) and a GTPase domain with extensive homology to the polypeptide chain elongation factor EF1α (Nagata *et al.*, 1984; Schirmaier and Philippsen, 1984).

Results

Arrest phenotype of the gst1 mutant

A new temperature-sensitive (*ts*) mutant of *S.cerevisiae* was isolated as described in Materials and methods. The strains YK21-02 and YK21-03 were derived from a backcross of the original isolate. In the second cross of YK21-02 with a wild-type C5051-3D, the diploid was temperature resistant and the *ts* phenotype segregated 2+:2−, indicating that it was a single and recessive mutation (*gst1-1*). When asynchronous cultures of the strain YK21-02 or YK21-03 were grown at 26°C or 30°C (permissive temperature) and shifted to 36°C (non-permissive temperature), budded cells accumulated (74% of the total cells in the case of YK21-02 and 64% in YK21-03), as shown in Figure 1A. Although the size of the buds and the percentage of budded cells seemed to be variable by genetic backgrounds, the terminal

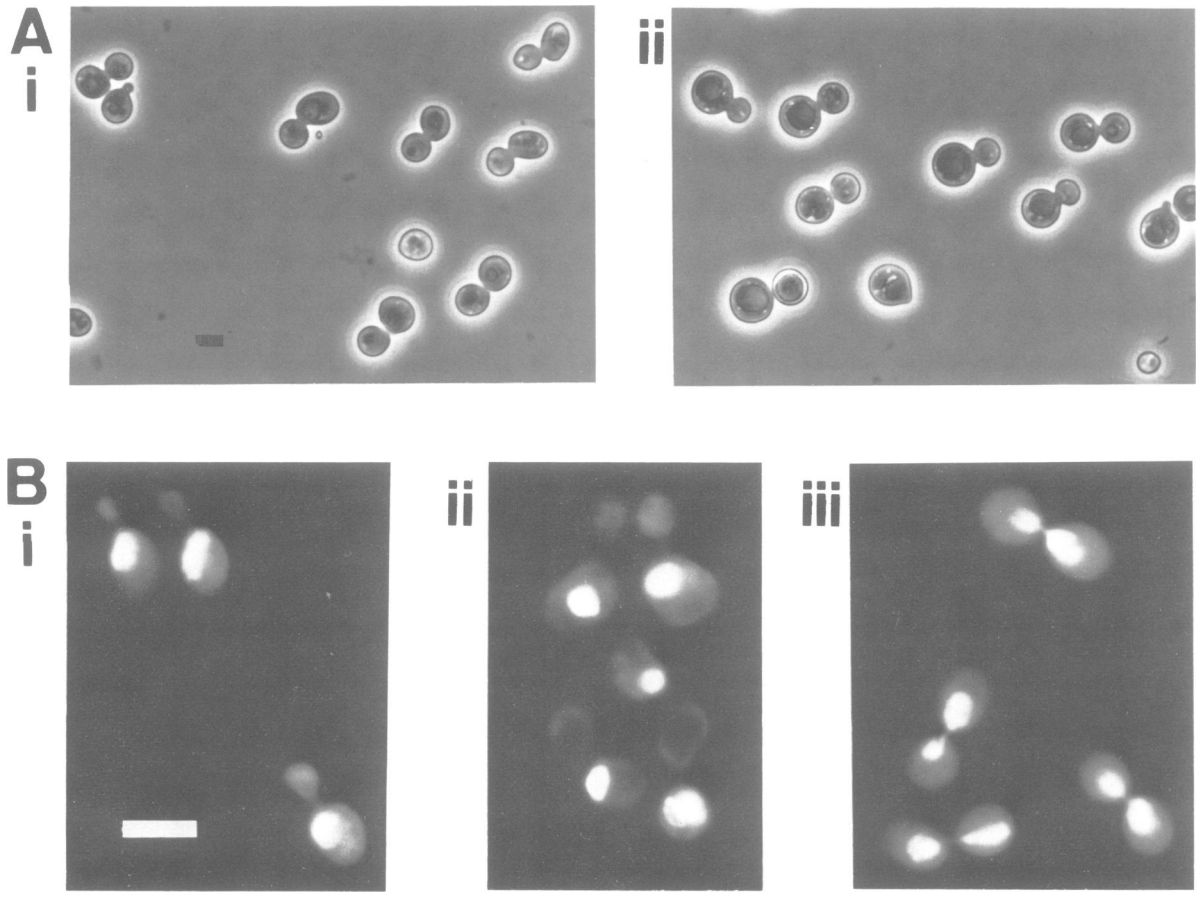

Fig. 1. Arrested morphology of the *gst1* mutant and location of the nuclei. (**A**) Cultures of the mutant cells were grown in YPD at 26°C and transferred to 36°C. At the following times after shift, photographs were taken: (**i**) *gst1-1* (strain YK21-02), 6 h; (**ii**) *gst1-1* (YK21-03), 6 h. (**B**) Mitochondrial DNA from yeast cells was removed as described in Materials and methods and nuclear DNAs were stained with DAPI. (**i**) Cells of *gst1-1* ϱ^0 (YK21-02 ϱ^0) incubated at 36°C for 6 h after shift. (**ii**) *gst1-1* ϱ^0 (YK21-02 ϱ^0), 36°C, overnight. (**iii**) *cdc7-1* ϱ^0 (124 ϱ^0), 36°C, 6 h. Bar = 5 μm.

morphology of the arrested cells appeared to be cell cycle specific.

In order to find at which stage of the cell cycle the mutant cells were blocked in the restrictive condition, we determined whether it was after the mating-pheromone-sensitive step or not. The α-mating factor arrests cells of *a* mating type in the late G_1 stage, before the initiation of DNA synthesis, and changes them to schmoo structures. Since the mating efficiency of the strain YK21-02 or YK21-03 was $<10\%$ (this phenotype was derived from *ste* of the original strain), we used the strain YK32-2C in which the phenotype of low mating efficiency segregated out in the second cross. After 3 h of treatment with α-factor, the cells were washed by filtration, transferred to a fresh medium (0 h, Figure 2Ai) and incubated at 37°C. Bud formation occurred and the buds grew bigger as the time increased (Figure 2Aii, iii). As a reciprocal experiment, arrested cells at the restrictive temperature (27°C, 3 h, Figure 2Bi) were mixed with α-factor and the culture was shifted to 26°C. Nuclear and cell divisions occurred and both mother and daughter cells changed to schmoos during 3 h (Figure 2Bii, iii). The cell number increased by 1.6-fold. Therefore, the execution point seemed to be after the mating-factor-sensitive step (post 'START').

DNA synthesis of mutant cells was measured as described in Materials and methods. Asynchronous cultures of the strain YK21-02 ϱ^0 missing its mitochondrial DNA were continuously labeled with [^{3}H]uracil at 26°C, and transferred to 36°C. After temperature shift, the DNA synthesis was substantially arrested, compared with the protein synthesis, which was followed by the incorporation of [^{35}S]methionine (Figure 3).

In the G_1-to-S phase transition of the cell cycle of *S.cerevisiae* two *cdc* genes, *cdc4* and *cdc7*, are well characterized. Temperature sensitivity of the strain *gst1* complemented either *cdc* mutant. The dumb-bell-shaped terminal morphology and defective DNA synthesis of *gst1* were similar to the phenotype of *cdc7*, except the location of nucleus. When the nuclear DNA of the strain YK21-02 ϱ^0 was fluorescently stained with DAPI, the nuclei appeared to locate near the junction of the buds (Figure 1B). In contrast, the arrested nucleus of *cdc7* migrated into the isthmus between the bud and the mother cell (Figure 1B; Hartwell *et al.*, 1973).

Isolation of plasmid capable of complementing gst1-1 mutation

A plasmid pYK801 capable of complementing the *gst1-1* mutation was isolated from gene libraries in multicopy vector YEp24 (Botstein *et al.*, 1979). The restriction map of the plasmid is shown in Figure 4. To localize the functional *GST1* gene, various DNA fragments were subcloned into a centromere vector YCp50 (Kuo and Campbell, 1983), which carried the yeast *URA3* gene, *ARS1* (*a*utonomously *r*eplicating *s*equence), *CEN4* (centromere 4) in pBR322 and

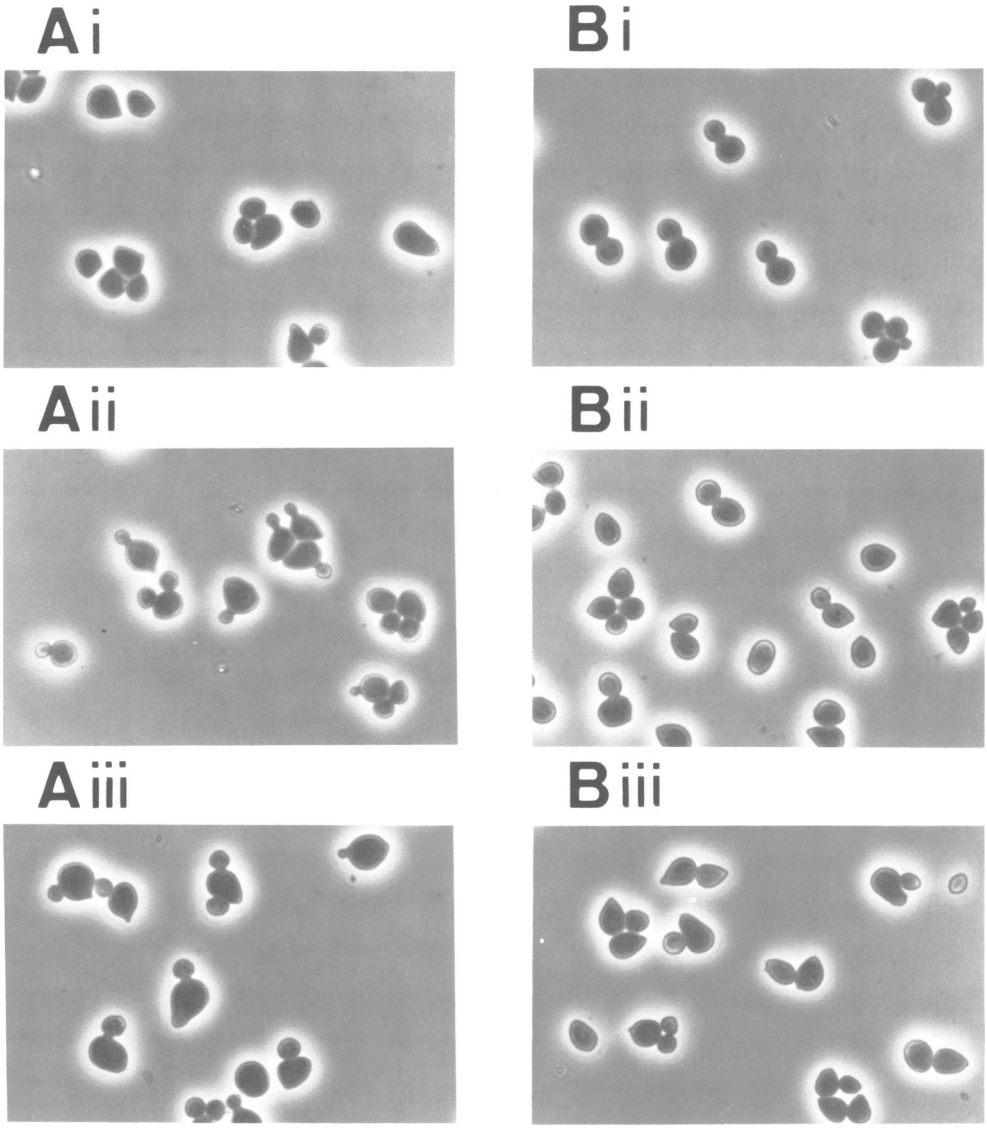

Fig. 2. Reciprocal experiment of temperature-shift and mating-factor treatment. **(A)** Cells of the strain YK32-2C were grown in YPD (pH 4) at 26°C and treated with 40 μg/ml α-factor for 3 h at 26°C. After washing α-factor by filtration, the culture was transferred to a fresh medium and grown further at 37°C. **(i)** 0 h, **(ii)** 2 h, **(iii)** 3 h after temperature shift. **(B)** Cells of YK32-2C were cultivated at 26°C and shifted to 37°C. After 3 h at restrictive temperature the cells were mixed with α-factor and transferred to 26°C to resume the growth: **(i)** 0 h, **(ii)** 2 h, **(iii)** 3 h incubation with α-factor.

behaved as a mini-chromosome in yeast. The resulting plasmids were introduced into YK21-02 and Ura^+ transformants were selected at 26°C. The temperature sensitivity of the transformants was checked by growth on a rich medium at 36°C. The complementing activity was localized within the 3.1-kb DNA region between the *Xba*I and *Pvu*II sites and the minimal requirement was the 1.3-kb region between the *Sal*I and the *Pst*I sites (Figure 4A), although the plasmid pYK825 carrying the 3.1-kb (*Eco*RI−*Pst*I) fragment partially complemented.

To confirm that the cloned DNA fragments contained the *GST1* gene itself and not an extragenic suppressor, the 4.6-kb (*Eco*RI−*Xho*I) fragment was inserted into an integration vector YIp5 (Scherer and Davis, 1979), which carried the *URA3* gene in pBR322. Since this hybrid, designated pYK821 (Figure 4B), cannot replicate autonomously in yeast, stable transformants arise only if the plasmid integrates into a chromosome by homologous recombination. To facilitate homologous recombination, the plasmid DNA was linearized with the restriction enzyme *Sal*I, the site of which was located in the middle of the gene and integrated into the chromosome (Orr-Weaver *et al.*, 1981). Ura^+ transformants were temperature-resistant recombinants. If they were mated with a wild-type strain (−+/+), only 5% of the spores turned out to be temperature sensitive upon meiosis, indicating that the cloned DNA was located at or close to the *gst1-1* mutation.

Mapping of the GST1 gene

To map the *GST1* gene, we performed the Southern blotting analysis to the yeast chromosomal DNAs fractionated in size by the orthogonal field-alternation gel electrophoresis (OFAGE) system (Carle and Olson, 1985), using pYK802 as a probe; the 8.9-kb *Eco*RI fragment of pYK801 carrying the *GST1* gene and pBR322 was self-ligated to make pYK802. The DNA probe hybridized to the top-most band to which the *TRP1* probe also hybridized, indicating that the cloned DNA was derived from chromosome 4 (data not shown).

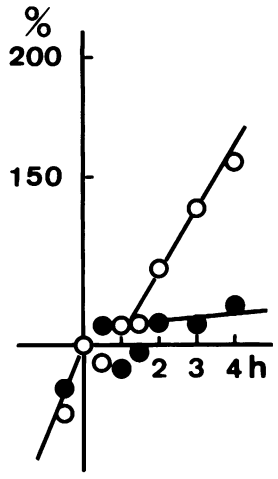

Fig. 3. DNA and protein synthesis. Assay procedure was described in Materials and methods. Results are expressed in percentages to the amount at the time of the temperature-shift. ○—○ protein; ●—● DNA.

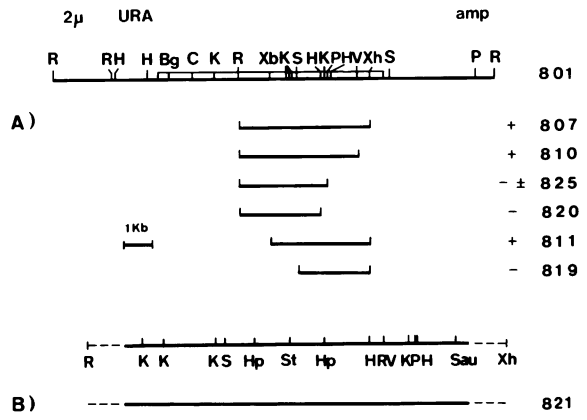

Fig. 4. Localization of the functional *GST1*. The restriction map of the plasmid pYK801 is drawn. YEp24 vector contains 2 μ-*ORI* and *URA3* on pBR322 (Botstein *et al.*, 1979). The yeast genomic DNA fragments have been cloned at the *Bam*HI site of the vector. The open box represents the insert. R, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; Xb, *Xba*I; S, *Sal*I; P, *Pst*I; V, *Pvu*II; Xh, *Xho*I.
(**A**) Complementation of *gst1* with various hybrid plasmids. DNA fragments from the insert of pYK801 were subcloned into YCp50 (Kuo and Campbell, 1983). Plasmid DNAs were introduced into *gst1* and Ura$^+$ transformants were selected at 26°C. Thermosensitivity of those transformants was checked at 36°C. pYK807: 4.6-kb (*Eco*RI−*Xho*I) fragment was cloned into YCp50. pYK810: DNA region between *Pvu*II site in the insert and *Nru*I site in the vector was deleted from pYK807. pYK825: 3.1-kb (*Eco*RI−*Pst*I) fragment and 2-kb (*Pst*I−*Hin*dIII) fragment of 2 μ-plasmid used as a linker, were inserted between *Eco*RI and *Hin*dIII sites of YCp50. pYK820: 2.8-kb (*Eco*RI−*Hin*dIII) fragment was cloned into YCp50. pYK811: 1.1-kb (*Eco*RI−*Xba*I) fragment in the insert of pYK807 was replaced with (*Eco*RI−*Xba*I) fragment containing *TRP1* region of YRp16 (Stinchcomb *et al.*, 1982). pYK819: 2-kb (*Eco*RI−*Sal*I) fragment of pYK807 was replaced with 2-kb (*Eco*RI−*Sal*I) fragment of YRp16.
(**B**) Plasmid structure for homologous integration. pYK821: 4.6-kb (*Eco*RI−*Xho*I) fragment was inserted between *Eco*RI and *Sal*I sites of YIp5 (Scherer and Davis, 1979). DNA sequence was determined (see Figure 6) in the region indicated by thick line. Hp, *Hpa*I; St, *Stu*I; RV, *Eco*RV; Sau, *Sau*3A.

Genetic evidence indicated that the *GST1* gene was located near the *CDC37* gene. In a cross of *gst1* with *cdc37*, the diploid was temperature resistant and after meiosis one out of 20 tetrads had a *ts*$^+$ recombinant, while other markers,

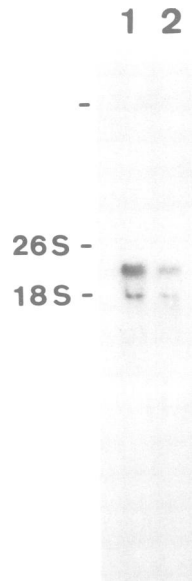

Fig. 5. Identification of *GST1* RNA transcript. A 10-μg sample of total RNA from strain DBY747*a* (**1**) or YNN27α (**2**) was denatured with glyoxal and resolved by 1.1% agarose gel electrophoresis, as described in Materials and methods. RNAs visualized by ethidium bromide staining were marked as size markers.

Trp and His segregated 2+:2−. Other *ts* mutants, *sec1*, *sec5* and *sec7*, mapped near *cdc37*, complemented with *gst1*. Therefore, the *GST1* gene may be a new *ts* gene near the *CDC37* gene on chromosome 4R.

RNA transcript

Total RNA was prepared from the wild-type strains DBY747*a* and YNN27α and a Northern blotting analysis was performed using the pYK812 DNA (pTZ18R containing the 1.3-kb *Kpn*I fragment from pYK801) as a probe. A major band was seen at 2.4 kb and a minor band appeared near 18S rRNA (Figure 5).

The nucleotide sequence of the GST1 locus

The nucleotide sequence of the cloned DNA fragment of the *GST1* gene and its flanking region was determined by dideoxy-chain termination method as shown in Figure 6. A predicted amino acid sequence (685 amino acids) for the long open reading frame (2055 bp) is also shown. The calculated mol. wt of the protein is 76 565. At several positions, including the sequence from −21 to −18 in the 5′ upstream region, there are TATA sequences. The nucleotide sequence downstream from the TAA termination codon contains sequences TAG···TAGT···TTT from +2170 to +2195, which may be associated with transcription termination and polyadenylation (Zaret and Sherman, 1982). A second potential *GST1* polyadenylation sequence, AATAAA is present at nucleotides +2139 to +2144 (Figure 6; Fitzgerald and Shenk, 1981; Bennetzen and Hall, 1982).

Homology with elongation factor 1α

The predicted *GST1* protein from the DNA sequence data is constituted from three domains. The domain I (codons 5−135) is rich in glutamine (30%), asparagine (16%) and tyrosine (15%), and highly conserved stretches of amino acids, QGGYQQ(Q)YNP, repeat about four times. In the

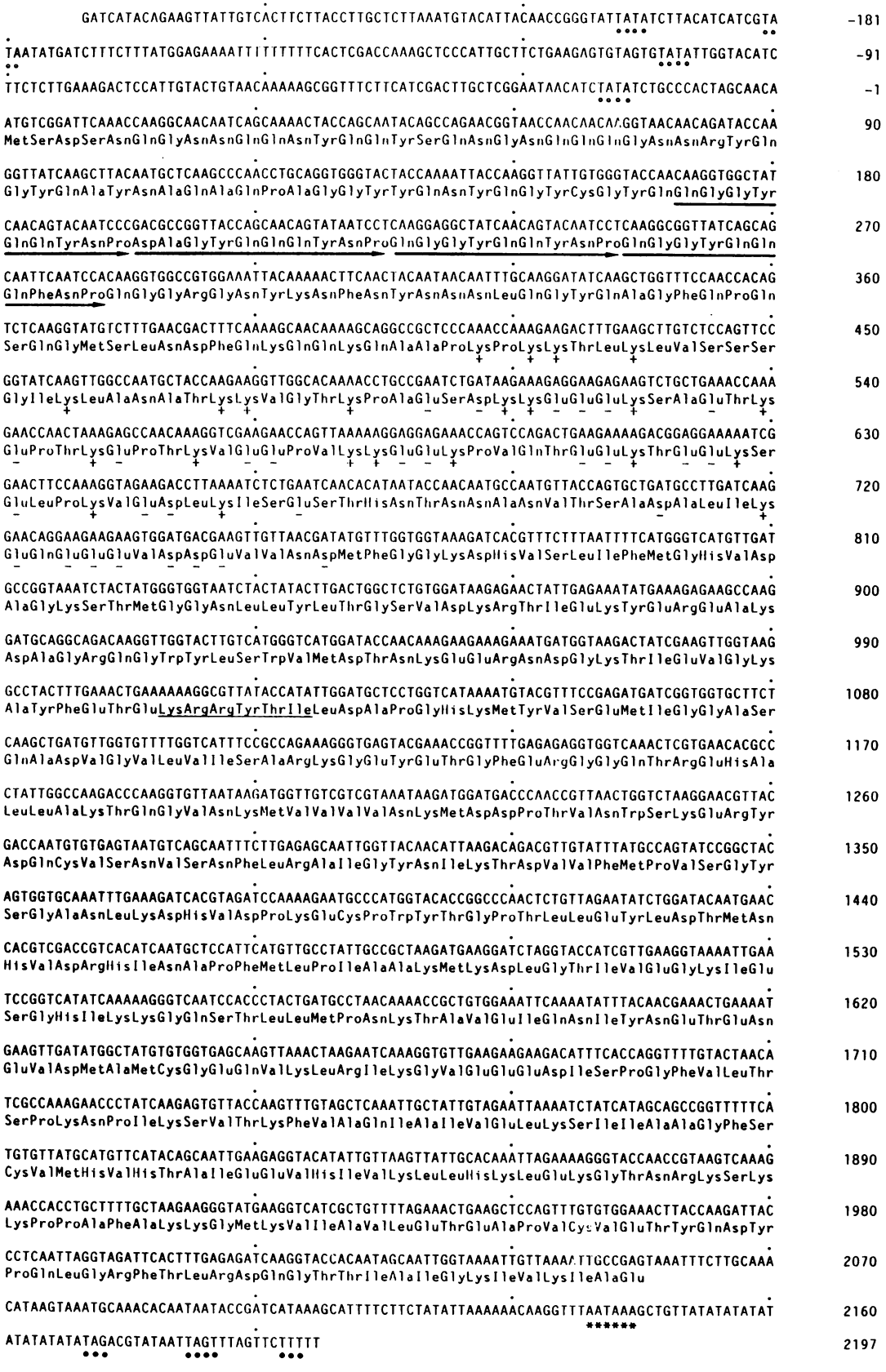

Fig. 6. Nucleotide sequence and predicted amino acid sequence of the *GST1* gene product. The coding sequence runs for 2055 nucleotides, which would encode a protein of 685 amino acids. The putative 'TATA' boxes are located at several positions marked by open circles. The potential signals for transcription termination and polyadenylation are indicated by closed circles and asterisks. The repetitive and highly conserved stretches of amino acids in domain I are shown by arrows. Basic or acidic amino acids in domain II are indicated as + or −. Comparison of amino acid sequence of domain III with EF1α is shown in Figure 7. The putative recognition site of A-kinase is underlined.

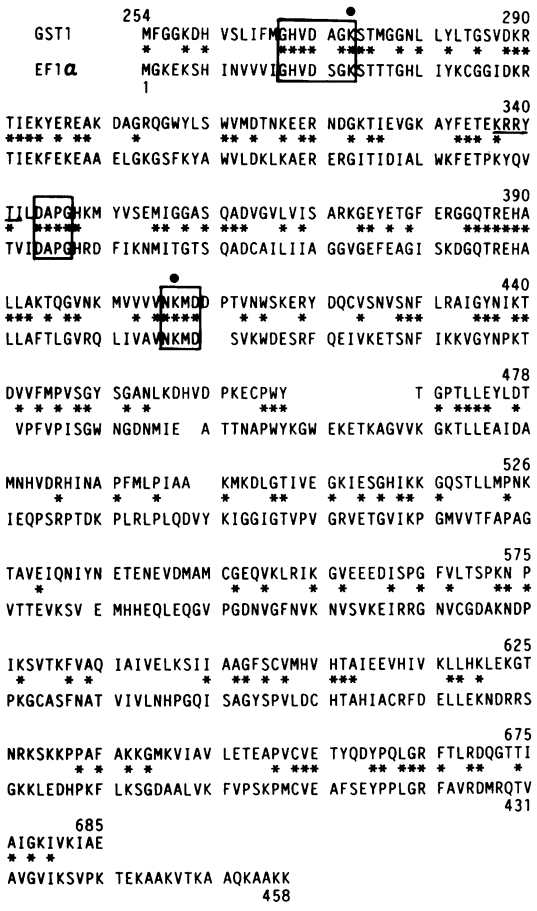

Fig. 7. Comparison of amino acid sequence of domain III of *GST1* with EF1α. Homologies between two sequences are indicated by asterisks. The region around 339 which might be recognized by A-kinase is underlined and the threonine at 341 might be phosphorylated. Three boxed regions specify consensus sequence elements in the GTP-binding domain (Dever *et al.*, 1987). Two lysine residues which might interact with GTP/GDP are marked by closed circles.

domain II (codons 139−249), acidic (especially Glu) and basic amino acids (mostly Lys) appear in turn as clusters. In the domain III which starts at codon 254 and ends at the carboxy terminus, extensive homology is found with yeast polypeptide chain elongation factor 1α (EF1α) (Nagata *et al.*, 1984; Schirmaier and Philippsen, 1984). The extent of homology with EF1α is 49% in the nucleotide sequence and 38% in the amino acid sequence (Figure 7). In particular, homology with the first half of EF1α is noteworthy; this is characteristic of a GTP-binding domain and GTPase activity center, as deduced from the X-ray crystallography of EF-Tu protein, an elongation factor of *Escherichia coli* (Jurnak, 1985). Three consensus sequence elements in the GTP-binding domain with distinct spacing (Dever *et al.*, 1987) are boxed in Figure 7. Two lysine residues at codons 273 and 407 might interact directly with GTP/GDP. Less homology is evident in the second half of EF1α, while a small part of the carboxy-terminal portion resumes some similarity between two sequences.

Furthermore, the sequence KRRYTI (codons 337−342) may represent a target site of cAMP-dependent protein kinase (A-kinase) (Cohen, 1985), which might phosphorylate the threonine residue at the position of codon 341.

Discussion

We have isolated many *ts* mutants using the selection described in Materials and methods. One group of these mutants accumulated budded cells after incubation at high temperature. Morphologically they are clearly different from 'START' mutants, which end up large unbudded cells. The new mutants proceed to the next stage and are arrested, like the *cdc7* mutant, with bud formation. The original selection could not be reproduced on the mutant described in this paper. It is not clear at this moment why we could collect such particular mutants. A CEN-plasmid was less stable at higher temperature and its copy number did not increase in the *gst1* mutant (unpublished observation).

The domain III of the *GST1* protein is strikingly similar to polypeptide chain elongation factor EF1α (Nagata *et al.*, 1984; Schirmaier and Philippsen, 1984). Since the stereochemical structure of the GTP-binding and GTPase domain of the EF-Tu protein of *E.coli* was already deduced from X-ray crystallography (Jurnak, 1985), we can superimpose the *GST1* protein on the EF-Tu protein with appropriate adjustments. Three consensus sequence elements for the GTP-binding domain in GTP-binding proteins are boxed in Figure 7 (Dever *et al.*, 1987). The region between the first and second consensus elements is known to interact with its effector. In this region the consensus sequence for the A-kinase target site is located. It is interesting to see whether *GST1* protein interacts with some factor(s) and if its binding is regulated by the phosphorylation.

In the signal transmission pathway, G-proteins, including *RAS* proteins localized at the membrane, modulate protein kinases. Subsequent phosphorylation of the target proteins is one of the critical events in the onset of S phase. Considering that *GST1* protein carries a potential target site for A-kinase, it would function epistatically to the kinase function. So, it is unlikely that the protein is located at the membrane like the authentic G-proteins. Also, the protein does not contain a Cys residue near the carboxy terminus, required for membrane localization (Powers *et al.*, 1986).

The amino acid sequence of *GST1* protein is not conclusive yet, because we only deduced this from the DNA sequencing data. We have not examined whether the gene contains intron sequences or not. However, the size of the 2.1-kb coding region, 0.1-kb 3′-untranslated region, 5′-untranslated region plus poly(A) chain appeared to be reasonable, compared with the size of the 2.4-kb mRNA as shown in Figure 5. Moreover the nucleotide sequence did not contain perfect consensus signals for splicing in yeast, GTATGT···TACTAAC···CAG (Schatz *et al.*, 1987). In the complementation experiment shown in Figure 4 one of the boundaries of the functional region should be beyond the restriction enzyme *Pst*I site and may be close to the boundary since the plasmid pYK825 complemented partially. In fact, the *Pst*I site is located close to the N terminus (codon 41) in the putative protein. We need further studies to clarify these undefined problems.

None the less, the fact that the tentative *GST1* protein, one of the essential genes for G_1-to-S transition, contains a potential A-kinase target site and GTPase domain would help in the analysis of the cell-cycle-specific events in eukaryotic cell proliferation. We are currently attempting to establish these findings biochemically and to see how they are regulated with respect to the cell cycle.

Materials and methods

Strains and genetic manipulations

E.coli JA221 (Beggs, 1978) was used for propagating plasmids and JM105 for M13 phage growth (Davis *et al.*, 1986). The strains of *S.cerevisiae* used in this study are the following: YK6-42, *a ste ade1 leu2 ura3 trp1 his3 cir*^{0}] (Kikuchi, 1983); C5674-3B, α *trp1 arg4 lys7*; YK21-02, α *gst1-1 ura3 trp1 his3* (YK6-42 *gst1-1* × C5674-3B); YK21-03, *a gst1-1 ura3 trp1 leu2 ade1 arg4* (YK6-42 *gst1-1* × C5674-3B); XMF2-28, α *sec1-1 trp1* (Nishizawa); XMF9-10, α *sec5-24 trp1* (Nishizawa); XMF3-6, α *sec7-1 trp1* (Nishizawa); DBY747, *a his3 ura3 leu2 trp1* (Matsui); YNN27, α *trp1 ura3 gal2* (Matsui); C5051-3D, *a his4 leu2 thr4 lys7*; YK32-2C, *a gst1-1 leu2 lys7 thr4 his* (YK21-02 × C5051-3D); SR672-1, *a cdc37-1 ura1 cyh2 gal2*; 124, *a cdc7-1*. The *cdc* mutants were derived from Yeast Genetic Stock Center at Berkeley.

Media, methods of mating, tetrad analysis and isolation of ϱ^0 strains were as described by Sherman *et al.* (1986). For α-factor treatment, the cells were grown in YPD (pH 4.0) and 40 μg/ml α-factor (Peptide Institute, Japan) were added at a cell density of 2 × 10^7/ml.

Plasmids and transformation

The YEp24 library was kindly provided by D.Botstein. Total DNA was partially digested with the restriction enzyme *Sau*3A and the fragments were inserted into the *Bam*HI site of YEp24 (Botstein *et al.*, 1979). Vectors used in this study were YCp50 (Kuo and Campbell, 1983), YIp5 (Scherer and Davis, 1979), YRp16 and YCp19 (Stinchcomb *et al.*, 1982), pJDB219 (Beggs, 1978) and pTZ18R (Pharmacia). Plasmid DNA was prepared as described previously (Kikuchi and Toh-e, 1986). Yeast transformation was performed by alkali ion method (Ito *et al.*, 1983). When linear DNA was used, tRNA (Sigma) was added as a carrier.

Isolation of gst1 mutant

The original aim of the mutant selection was to isolate *ts* mutants in which proteins acting on the centromere were defective, as described in the Introduction. For this selection procedure, the following plasmids were constructed. Plasmid pYK2068 was made by inserting the 2.4-kb *Bgl*II fragment containing *CEN4* of YCp19 (Stinchcomb *et al.*, 1982) into the *Bam*HI site of pJDB219. Plasmid pJDB219 harbored the entire yeast 2 μ-plasmid sequence (but the *FLP* gene was destroyed) on pMB9 vector along with a partially defective *LEU2* as a selective marker (Beggs, 1978). Since the 5′ upstream region of the *LEU2* was defective, the expression of Leu$^+$ phenotype was so low that transformants were Leu positive only when cells contained multicopies of this plasmid (Erhart and Hollenberg, 1983). Plasmid pYK2090 was constructed by inserting the same *Bgl*II fragment of YCp19 containing *CEN4* into pYK2029 which carried the *FLP* gene and *URA3* as a selective marker as described in Kikuchi (1983).

The yeast strain YK6-42 was mutagenized with ethyl methanesulfonate (8% survivals), grown in a minimal medium and cells were transformed with both plasmids pYK2068 and pYK2090. After 6 days of incubation at 34°C, ~8000 Ura$^+$ Leu$^+$ transformants were obtained. Most colonies were very tiny but 27 were relatively large. Fourteen out of 27 clones were *ts* mutants. One of these *ts* mutants was crossed with a wild-type strain C5674-3B, and YK21-02 and YK21-03 carrying the *gst1-1* allele were obtained.

Assays for macromolecular synthesis

The procedure for the measurement of DNA and protein synthesis was essentially as described by Johnston and Game (1978), except that the cells were continuously labeled with 20 μCi/ml [5,6-^{3}H]uracil (46 Ci/mmol; Amersham) or 6 μCi/ml [^{35}S]methionine (300 Ci/mmol; Amersham) in YPD medium, supplemented with 20 μg/ml adenine.

DAPI staining of nuclear DNA

Samples of 1 ml of ϱ^0 cells were removed at various times from a culture grown at 36°C (i.e. the non-permissive temperature). The cells were collected by centrifugation, washed once with 25% ethanol, 15 mM $MgCl_2$ and suspended in the same solution. They were allowed to fix at room temperature for at least 30 min and washed twice with cold water by centrifugation. The fixed cells were suspended in 0.5 μg/ml DAPI (4′,6′-diamidino-2-phenylindole, Sigma) in water and viewed through an Olympus DApo 100 UV lens on an Olympus BH2 microscope equipped for epifluorescence, and photographed with Kodax Tri-X film.

Orthogonal field-alternation gel electrophoresis (OFAGE)

The samples of yeast DNA were prepared by the embedded-agarose procedure described in Carle and Olson (1985). We designed and used a simplified model of the apparatus for OFAGE (Carle and Olson, 1984). The DNA samples were electrophoresed through 1.5% agarose gel (10 × 10 cm) in 45 mM Tris, 45 mM boric acid and 0.5 mM EDTA (pH 8.0), at 200 V with a switching interval of 50 s, for 20 h at 14°C. The gel was stained with 0.5 μg/ml ethidium bromide, extensively washed with deionized water and photographed on a UV transilluminator.

Southern and Northern analysis

Southern blotting analysis was performed as described previously (Kikuchi, 1983) except that the labeled probe was prepared with [^{32}P]dCTP using the Multiprime™ DNA labeling system (Amersham). For Northern analysis, total RNA was prepared as described by Maniatis *et al.* (1982). The RNA sample was incubated wtih 50% dimethylsulfoxide, 10 mM sodium phosphate (pH 7.0), 1 M glyoxal at 50°C for 60 min. Then the sample was fractionated by 1.1% agarose gel electrophoresis in 10 mM sodium phosphate (pH 7.0). Without any pretreatment, the gel was blotted to nitrocellulose filters. The bands of ribosomal RNAs were visualized by ethidium bromide staining and used as size markers.

DNA sequencing

Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using an M13-sequencing kit (Takara Shuzo Co., Kyoto). DNA fragments were cloned into M13 mp18 or mp19. Reaction products were resolved by electrophoresis through 8% acrylamide gels under denaturing conditions.

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