Size Selection Identifies New Genes That Regulate *Saccharomyces cerevisiae* Cell Proliferation

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ABSTRACT

A centrifugation procedure to enrich for enlarged cells has been used to isolate temperature-sensitive *cdc* mutants of the yeast *Saccharomyces cerevisiae*. Among these mutants are strains containing mutations that arrest proliferation at the regulatory step start. These new start mutations define two previously unidentified genes, *CDC67* and *CDC68*, and reveal that a previously identified gene, *DNA33* (here termed *CDC65*), can harbour start mutations. Each new start mutation permits significant biosynthetic activity after transfer of mutant cells to the non-permissive temperature. The *cdc68-1* start mutation causes arrest of cell proliferation without inhibition of mating ability, while the *cdc65-1* and *cdc67-1* mutations inhibit zygote formation and successful conjugation. The identification of new start genes by a novel selection procedure suggests that the catalog of genes that influence start is large.

GENETIC and physiological studies show that cell proliferation by the budding yeast *Saccharomyces cerevisiae* is regulated in the pre-replicative interval of the cell cycle (PRINGLE and HARTWELL 1981; WHEALS 1987); this regulatory step has been termed start (HARTWELL 1974). The performance of start, and thus the proliferation of yeast cells, is responsive to the biosynthetic status of the cell (WHEALS 1987). Indeed, the initiation of a new round of cell division is correlated with growth of a cell to what can be measured as a threshold cell size (the "critical size"; JOHNSTON, PRINGLE and HARTWELL 1977). Furthermore, limitation of biosynthesis by starvation for certain required nutrients can prevent the performance of start and bring about a regulated cessation of proliferation with cells arrested at start (HARTWELL 1974; JOHNSTON, PRINGLE and HARTWELL 1977; PRINGLE and HARTWELL 1981). In addition to this form of start regulation, particular effector molecules such as yeast mating pheromones can modulate start independently of biosynthetic activity (BÜCKING-THROM *et al.* 1973).

The control of yeast cell proliferation has been analyzed genetically, in part through the identification of conditional mutations that inhibit the performance of start (HARTWELL 1974; PRINGLE and HARTWELL 1981; WHEALS 1987). Under nonpermissive conditions cells harboring these mutations complete an ongoing cell-division cycle but are unable to initiate a new cell cycle, and thus accumulate at the regulatory step start. These start mutations have been grouped into two categories: mutations that bring about arrest of cell proliferation at start most likely as a secondary consequence of impairment of certain biosynthetic activities, and other mutations that have little effect on biosynthetic activity (REED 1980). Some start mutations of the latter type have been found to affect the pheromone response pathway, or components of the cell-cycle regulatory mechanism itself. For example, mutations in the *GPA1* gene, encoding the α subunit of a yeast G protein that is a transducer of the mating pheromone response, cause start arrest (JAHNG, FERGUSON and REED 1988). Other start mutations in this category affect the *CDC28* gene product, a protein kinase (LORINCZ and REED 1984) that is part of a high-molecular-weight complex required to initiate a new cell cycle (WITTENBERG and REED 1988). Certain other mutations of this type that affect start have been identified in a directed way, based on interactions with previously characterized genes (HADWIGER *et al.* 1989; REED *et al.* 1988); in some cases the products of the genes harboring these mutations have been shown to interact (HADWIGER *et al.* 1989). These genes and others known to affect start probably encode only a subset of the components involved in start regulation. It should therefore be profitable to extend the search for mutations that influence start without significant inhibition of biosynthetic activity.

The responsiveness of proliferation control to biosynthetic activity suggests that new start mutations sought without consideration of this particular rela-

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tionship will in most instances affect biosynthetic activities that influence start, rather than components of the start regulatory apparatus (see WHEALS 1987). However, all mutations that differentially inhibit the performance of start (primarily or only secondarily) produce the same basic phenotype: mutant cells accumulate at start, in the unbudded phase of the yeast cell cycle. Therefore other criteria, most notably the necessity for start-arrested mutant cells to undergo growth-related activities such as conjugation, have been used to identify interesting start mutations (REED 1980).

Here we describe the isolation and characterization of new start mutations that cause arrest of cell proliferation but still allow significant biosynthetic activity to continue. This work relied on a selection scheme designed to enrich for mutant cells that become enlarged and thus continue biosynthetic activity upon incubation at the nonpermissive temperature. Genetic mapping and molecular cloning indicate that the new mutations reported here define two previously unidentified genes, and show that a third gene harbors start mutations. Therefore three previously unsuspected genes are shown to affect start. Perhaps more significantly, the procedures used here identified mutations in known *cdc* genes but did not yield new mutations in well-known start genes, suggesting that the catalog of genes that function primarily to affect start may be large.

MATERIALS AND METHODS

Strains and growth media: The haploid strain GR2 (*MAT***a** *his6 ura1*) has been described (JOHNSTON and SINGER 1978). Strain 21R (*MAT***a** *ade1 leu2-3,112 ura3-52*; JOHNSTON and HOPPER 1982) was obtained from J. E. HOPPER and used to construct transformable strains. Strain Sc25k-13 (*MATα ade1 leu2-3,112 ura3-52 KEX1::LEU2*; DMOCHOWSKA *et al.* 1987) was used for genetic mapping. Strain XJB3-1B (*MATα met6*) was the *MATα* tester strain, and strains 47S-104A (*MAT***a** *cdc64-1 leu1*) and 47S-7 (*MAT***a** *cdc62-1*) (BEDARD, JOHNSTON and SINGER 1981) were reference strains in quantitative mating reactions (BEDARD, JOHNSTON and SINGER 1981; BEDARD *et al.* 1984). Yeast cells were routinely grown in YM1 complex medium (HARTWELL 1967) or YNB minimal medium (JOHNSTON, PRINGLE and HARTWELL 1977). Similar solid complex (YEPD) or synthetic media (HARTWELL 1967) were also used. *Escherichia coli* strain RR1 was obtained from J. E. HOPPER and used for plasmid isolation and maintenance. *E. coli* cells were grown in YT medium (MILLER 1972).

Assessment of cellular parameters: Yeast cell concentrations were determined using an electronic particle counter (Coulter Electronics, Hialeah, Florida). Before counting, cells were fixed using formalin and sonicated briefly (HARTWELL 1970). Cell morphology was assessed by direct microscopic examination. Rates of protein and RNA synthesis were estimated by a pulse-labeling procedure described previously (HANIC-JOYCE, JOHNSTON and SINGER 1987). DNA content was quantified by a modified diphenylamine procedure as described (STORMS *et al.* 1984).

Mutant isolation: Stationary-phase cells of strain GR2 were mutagenized by exposure to ethylmethane sulfonate (EMS, 1:20; Sigma) for 3 hr (Fink 1970) to yield 10–50% survival, then diluted 60-fold into replicate volumes of YM1 liquid medium and grown to stationary phase at 23°. For mutant enrichment these stationary-phase cultures were first diluted into fresh YM1 medium and incubated at 23°; the resulting exponential-phase cultures were then transferred to 36° and incubated for 3 hr. Reconstruction experiments using strains bearing previously characterized start mutations showed that this incubation time was long enough to allow temperature-sensitive start mutants to arrest cell division and grow significantly, but short enough to avoid the loss of cell viability characteristic of many mutants incubated for prolonged periods under nonpermissive conditions (see BEDARD, JOHNSTON and SINGER 1981). Cells were then harvested by centrifugation, washed once with phosphate buffer, and sonicated briefly to separate cells; a concentrated suspension of 10^7 of these cells was layered onto 10 ml of a colloidal suspension of glass beads, prepared by diluting Ludox HS30 (DuPont), a 30% suspension of colloidal glass beads, to 17% with 0.1 × YNB medium, adjusting to pH 8.0, and autoclaving. This suspension forms a gradient upon centrifugation (SHULMAN, HARTWELL and WARNER 1973), and after centrifugation at 42,000 × *g* for 10 min most of the cells had migrated sufficiently far into the tube to form a wide band about 1.5 cm from the bottom of the 10-cm tube. Cells were not centrifuged to their equilibrium density. With a U-shaped Pasteur pipette, samples of the gradient were removed from the area above the main band of cells, diluted and spread on YEPD solid medium. Replicas of the resultant colonies were incubated on YEPD medium at 23° and at 36° to identify colonies of temperature-sensitive cells.

Genetic analysis: Mutant isolates were analyzed by standard genetic procedures (MORTIMER and SCHILD 1981). Noncomplementation of new recessive temperature-sensitive mutations was verified by temperature sensitivity, on solid medium, of diploids selected by complementation of auxotrophies.

Mating assay: Conjugation by arrested mutant cells was assessed as described (BEDARD *et al.* 1984). In brief, actively dividing *MAT***a** mutant cells were transferred from 23° to 36° and incubated for 3.5 hr. These cells were then mixed with an equal number of cells of tester strain XJB3-1B, the cell mixture was collected on a nitrocellulose filter, and the filter disc bearing the cells was incubated on YEPD medium for a further 3 hr at 34°. These cells were then suspended in YM1 medium and immediately spread on solid selective medium to select for diploids on the basis of complemented auxotrophies. For comparison, the mating-proficient start-mutant strain 47S-7 and the mating-incompetent start-mutant strain 47S-104A were included in mating experiments. To ensure that mutant cells had remained viable during the incubations at nonpermissive temperatures, the number of viable mutant cells within the mating mixture also was assessed after plating the mating mixture on medium that would select against growth of the tester strain. Mating was also quantified with all incubations at 23°.

Molecular cloning: Genes identified by mutation were cloned by complementation (ROTHSTEIN 1986; ROSE 1987). For transformation, mutant strains were twice backcrossed to a transformable strain. In all cases, 10–30 μg of yeast genomic library DNA were used to transform mutant cells to both temperature resistance and uracil prototrophy by the method of HINNEN, HICKS and FINK (1978). The YEp24-based library R114 was kindly provided by D. BOTSTEIN. Restriction analysis was performed as described (MANIATIS, FRITSCH and SAMBROOK 1982). Probes were prepared using a Random Primed DNA Labelling Kit (Boehringer Mann-

heim) and [^{32}P]ATP, a gift from J. HOFMAN (Department of Biochemistry, Dalhousie University). Chromosomal assignments of cloned sequences were determined by hybridization to blots of intact yeast chromosomes separated by CHEF (contour-clamped homogeneous electric field) electrophoresis (CHU, VOLLRATH and DAVIS 1986) and transferred to Genescreen membranes (New England Nuclear) by L. SCHALKWYK (Department of Biochemistry, Dalhousie University), using the method of REED and MANN (1985). Southern analyses were performed as described (MANIATIS, FRITSCH and SAMBROOK 1982).

Construction of integration plasmids: To integrate the cloned *CDC65* insert, a *Bam*HI fragment was first removed from the complementing insert sequence in plasmid pLE9-3 (Figure 3), which was then self-ligated. The resultant plasmid was linearized with *Bgl*II to direct integration to the homologous chromosomal locus and thus mark this locus with the plasmid-borne *URA3* gene (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981). Transformed *cdc65-1* haploid cells were grown under nonselective conditions to identify a stable integrant containing this otherwise-episomal plasmid. Homologous integration of insert sequences was confirmed by Southern analysis (Figure 3). Analysis of 33 tetrads demonstrated that the *URA3* gene and the *cdc65-1* mutation co-segregated.

Integration of the *SCC65* sequence was accomplished by subcloning into plasmid YIp5 a 4.4-kb *Hin*dIII fragment from plasmid pLE3-3 (Figure 3) containing insert and vector sequences. The resulting plasmid was cleaved with *Bgl*II to direct integration, and homologous integration in *cdc65-1* haploid cells was confirmed by Southern analysis (Figure 3). Tetrad analysis revealed that the *SCC65* locus, as marked by *URA3*, did not segregate with the *cdc65-1* mutation (8 PD:8 NPD:32 TT).

For the *CDC67* gene a 2-kb *Hin*dIII fragment from the insert in p67-13E (Figure 3) was subcloned into YIp5, and homologous integration of this plasmid into *cdc67-1* haploid cells was directed by cleavage within the insert sequence using *Xba*I, and confirmed by Southern analysis (Figure 3). Analysis of 19 tetrads demonstrated that the plasmid-borne *URA3* gene co-segregated with the *cdc67-1* mutation.

For the cloned *CDC68* gene, a 1.7-kb *Hin*dIII fragment from the insert of plasmid pSC2-1 was subcloned into YIp5. This plasmid was integrated into the chromosome of haploid *cdc68-1* cells, and shown by Southern analysis to be integrated at the insert locus (Figure 3). Analysis of 26 tetrads showed cosegregation of the plasmid-borne *URA3* gene and the *cdc68-1* mutation.

RESULTS

Mutant isolation: Actively proliferating mutagenized cells were transferred from 23° to the nonpermissive temperature of 36° and incubated for 3 hr. This protocol allowed the enlargement through continuing biosynthetic activity of the rare mutant cells that continue to grow when blocked in cell division at the nonpermissive temperature. These cell suspensions were then resolved by centrifugation through colloidal glass beads to separate cells by size. After centrifugation, the cells in regions of the centrifuge tube expected to contain larger-than-normal cells were tested for temperature sensitivity by replica plating and by growth in liquid medium, and assessed for phenotype.

TABLE 1

Reconstruction experiment for mutant isolation

cdc mutation	Enrichment factor		
	Fraction A	Fraction B	Fraction C
cdc4-1	87	48	57
cdc8-3	416	327	238
cdc14-1	≤1	32	26
dna40-1	70	11	3
dna42-1	170	270	112
cdc28-4	140	88	167
cdc64-1	≤1	≤1	≤1
cdc25-1	≤2	≤5	≤1

In separate experiments *cdc* mutant cells were mixed 1:1000 with wild-type cells of strain GR2 and centrifuged through colloidal glass beads as described in MATERIALS AND METHODS. Following centrifugation three adjacent 1-ml fractions were removed from above the main band of cells, with fraction C the one nearest to the main band. The proportion of temperature-sensitive cells in each fraction was determined by replica-plating procedures, and expressed relative to the input proportion as the enrichment factor.

The efficacy of this centrifugation method for mutant isolation was determined by reconstruction experiments. Suspensions of wild-type cells were seeded with small numbers of cells harboring previously characterized *cdc* mutations, and subjected to the enrichment procedure. As shown in Table 1, in these reconstruction experiments the procedure significantly enriched for a variety of cell-cycle mutants. Also included in these reconstruction experiments were two negative controls: *cdc64-1* mutant cells that arrest cell proliferation at start and do not increase in size when arrested (BEDARD, JOHNSTON and SINGER 1981), and *cdc25-1* start-mutant cells that are defective in nutrient sensing (ROBINSON *et al.* 1987) and also do not increase in size (IIDA and YAHARA 1984). For each of these mutant cells there was no enrichment by this selection procedure (Table 1). As expected, the procedure precludes the efficient isolation of mutants severely compromised in growth abilities.

Using EMS-mutagenized cultures, approximately 0.1% of colonies derived from cells in the region above the main band of cells in each centrifuged sample showed some degree of temperature sensitivity. Of temperature-sensitive colonies, approximately 10% were composed of cells that after cessation of cell division at the nonpermissive temperature displayed a uniform cell morphology, diagnostic of a cell-division-cycle (*cdc*) mutant (HARTWELL, CULOTTI and REID 1970; HARTWELL *et al.* 1973). Some of these isolates are characterized below. Other colonies contained mutant cells that produced phenotypes reminiscent of cell lysis (*cly*) mutants (MORTIMER and HAWTHORNE 1973): these mutant cells upon incubation at the nonpermissive temperature became large and granular in appearance without acquiring a common terminal phenotype, and cell lysis was evident. These mutant cells were not studied further.

TABLE 2

Complementation analysis

New mutant alleles	Terminal phenotype[a]
cdc14 (2 alleles)	Large budded cells
dna40	Large budded cells
dna42	Large budded cells
cdc24 (4 alleles)	Large unbudded cells
cdc22	Unbudded and small-budded cells
cdc60-2	Unbudded cells
cdc65-1 (an allele of *dna33*)	Unbudded cells
cdc66-1	Unbudded cells
cdc67-1	Unbudded cells
cdc68-1	Unbudded cells

[a] Morphology of mutant cells after arrest of proliferation by incubation at the nonpermissive temperature.

TABLE 3

DNA content of arrested mutants

Mutation	Absorbance/10^8 cells[a]		Relative DNA content[b]
	0 h	6 h	
cdc65-1[c]	0.446	0.323	0.72
cdc67-1	0.232	0.176	0.76
cdc68-1	0.255	0.199	0.78
cdc35-1[d]	0.233	0.176	0.76

[a] DNA content, measured spectrophotometrically as described in Materials and Methods, was determined at the time of transfer (0 h) and after 6 h incubation of mutant cells at the nonpermissive temperature.

[b] The average DNA content of cells after arrest of proliferation (6 h), divided by the average DNA content of proliferating cells (0 h). Because individual cells in a G_1-arrested population have an unreplicated complement of DNA, while individual cells in a proliferating population have DNA contents that range from an unduplicated to a fully duplicated complement of DNA, the relative DNA content of G_1-arrested cells expressed in this way should be less than 1.0.

[c] This *cdc65-1* mutant is a homozygous diploid.

[d] For comparison, a previously characterized G_1-arrest mutant (*cdc35-1*; PRINGLE and HARTWELL 1981) was also analyzed.

Genetic analysis: Interesting mutants with uniform terminal morphologies at the nonpermissive temperature were subjected to genetic analysis. In several isolates more than one temperature-sensitive mutation was present; in some cases each of these mutations individually produced only a random arrest in the cell cycle as judged by cell morphology. Genetic segregants containing only a single recessive mutation producing a common terminal phenotype were chosen for further study.

Single mutations producing *cdc* phenotypes were tested by complementation against other recessive temperature-sensitive mutations, including *cdc* mutations. Included in these tests were strains bearing mutations in the *CDC1–CDC37*, *CDC39*, *CDC46*, *CDC47* (PRINGLE and HARTWELL 1981; WHEALS 1987), and the *CDC60*, *CDC62*, *CDC64* (BEDARD, JOHNSTON and SINGER 1981) and *CDC63* (*PRT1*; HANIC-JOYCE 1985; HANIC-JOYCE, SINGER and JOHNSTON 1987) genes. Also included in the complementation analysis were mutations in other genes producing *cdc* phenotypes, such as *DBF1-DBF4*, *DBF6*, *DBF7*, and *DBF9* (JOHNSTON and THOMAS 1982), in genes differentially affecting DNA metabolism, such as *DDS1–DDS6* (JOHNSTON and GAME 1978) and *DNA1–DNA60* (DUMAS *et al.* 1982), and in genes differentially affecting RNA metabolism, such as *RNA1–RNA9* and *RNA11* (HARTWELL, MCLAUGHLIN and WARNER 1970). As shown in Table 2, these complementation tests indicated that some of the newly isolated temperature-sensitive mutations were new alleles of previously identified complementation groups.

The isolation by this procedure of new mutations in certain previously identified genes (Table 2) verified the efficacy of the isolation procedure. For example, new mutations were readily obtained in the *CDC14* and *CDC24* genes; previously characterized mutations in each of these genes allow significant cell enlargement at the nonpermissive temperature (JOHNSTON, PRINGLE and HARTWELL 1977). New mutations were also obtained in three *DNA* genes; these genes were originally identified by mutations that inhibit DNA synthesis more severely than the accumulation of cell mass (DUMAS *et al.* 1982), and would thus be expected to allow significant cell enlargement at the mutationally induced block. Moreover, the *cdc* phenotypes produced by the new mutations in these *DNA* genes show that these genes may have specific effects on the cell cycle.

Several new mutations produced an unbudded phenotype characteristic of start mutations (Table 2). One of these mutations was allelic with *cdc60-1*, previously shown to block start (BEDARD, JOHNSTON and SINGER 1981). This mutation was not characterized further. A second new mutation, *cdc65-1*, was allelic with *dna33*, a mutation that had been shown to affect DNA replication but that had not been characterized for effects on the cell cycle (DUMAS *et al.* 1982). Finally, three of the new mutations complemented every tester mutation, and thus defined the genes *CDC66*, *CDC67*, and *CDC68*. Each of these three new mutations, plus *cdc65-1*, caused cells at the nonpermissive temperature of 36° to arrest proliferation within one cell cycle. In each arrested population 80–90% of cells were unbudded, suggesting that the arrested cells were in the G1 (prereplicative) interval of the cell cycle (HARTWELL 1974). The DNA contents of arrested cells confirmed G1 arrest for *cdc65-1*, *cdc67-1*, and *cdc68-1* mutant cells (Table 3). This G1 arrest was not accompanied by significant cell deformation (data not shown).

The *cdc66-1* mutation produces an unusual type of arrest, and will be the subject of another report.

Start arrest: Unbudded G1 arrest suggested that each of these new mutations inhibited the performance of start. To ascertain the relationship between

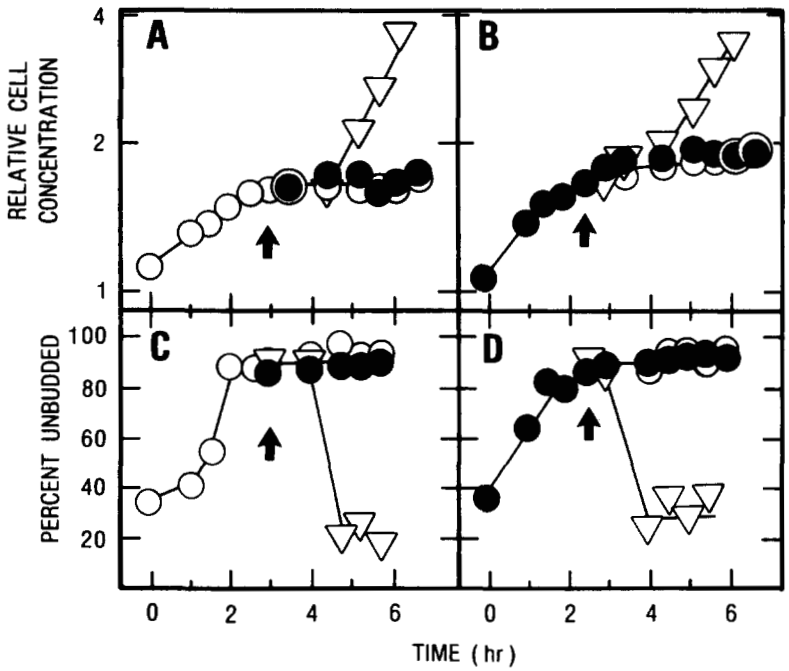

FIGURE 1.—Order-of-function analysis relating start and the *cdc65-1* block point. *Panels A and C*: mutant cells proliferating at 23° were exposed to α-factor for 3 hr, then transferred to medium without α-factor and incubated at 23° to test for recovery from α-factor treatment, or at 36° to test for cell division in the presence of the *cdc65-1* block. *Panels B and D*: mutant cells proliferating at 23° were transferred to 36° and incubated 2.5 hr, then returned to 23° and incubated to test for recovery from the temperature-induced block, or returned to 23° and incubated along with α-factor to test for cell division in the presence of that start inhibitor. Symbols: △, cells at 23°; ●, cells at 36°; ○, cells at 23° in the presence of α-factor.

start and the cell-cycle arrest point for each mutation, order-of-function analysis (HEREFORD and HARTWELL 1974) was carried out using the yeast mating pheromone α-factor as an authentic inhibitor of start (BÜCKING-THROM *et al.* 1973).

The results of an order-of-function experiment for the *cdc65-1* mutation are shown in Figure 1. Mutant cells of mating type **a** (requisite for α-factor sensitivity) were first arrested at start at the permissive temperature by treatment with α-factor (Figure 1, A and C). Further incubation in fresh medium without α-factor but at the nonpermissive temperature of 36° resulted in no change in the proportion of unbudded cells and no cell division, indicating that cells arrested in the cell cycle by mutation were blocked at or after the α-factor-sensitive step. In the reciprocal experiment (Figure 1, B and D), mutant cells were first arrested at the temperature-sensitive block by incubation for 2.5 hr at 36°. Further incubation of these cells at the permissive temperature but in the presence of α-factor also resulted in no change in the proportion of unbudded cells and no cell division, indicating that cells arrested by the mutation were blocked at or before the α-factor-sensitive step. Taken together these reciprocal-shift experiments indicate that the *cdc65-1* mutation blocks cells at a step interdependent with the α-factor-sensitive step; by this criterion the *cdc65-1* mutant cells arrest at start. Similar results from reciprocal-shift experiments were obtained for strains harboring the *cdc67-1* and *cdc68-1* mutations (data not shown). Thus each of these new mutations arrests cells at start.

The start-arrest phenotype for *cdc65-1* mutant cells suggested that the allelic *dna33* mutation, characterized only to prevent continued DNA replication (DUMAS *et al.* 1982), probably affected DNA replication by blockage of start, an event required for initiation of DNA synthesis (HEREFORD and HARTWELL 1974). In fact the *dna33* mutation, backcrossed into the GR2 genetic background, produced a modest (70%) unbudded-cell arrest upon transfer to 36° (data not shown). It is thus more informative to continue to refer to our new start mutation as *cdc65-1*, and to the allelic *dna33* mutation as *cdc65-33*.

Biosynthetic activity in arrested mutant cells: The mutant-isolation scheme used here was designed to enrich for enlarged cells. Each of the mutations did, in fact, lead to large unbudded cells (data not shown), and to marked cell enlargement (turbidity per cell) at the nonpermissive temperature (Figure 2, A, B, and C). To determine the effects of each start mutation on biosynthetic activity more directly, rates of protein and RNA synthesis were determined by pulse-labeling procedures. The rates of incorporation of radiolabeled precursors for protein and RNA by mutant cells were then normalized to the incorporation rates exhibited by related wild-type cells under the same conditions. In this way the effects on biosynthetic activity of a particular mutation could be distinguished from other effects on label incorporation caused by transfer to the nonpermissive temperature (HANIC-JOYCE, JOHNSTON and SINGER 1987). As shown in Figure 2, D, E, and F, each of the new start mutations permitted significant rates of incorporation upon transfer to the nonpermissive temperature. The *cdc65-1* mutation caused the greatest impairment of continued biosynthetic activity, but even these mutant cells maintained 20–40% of the control rate of precursor incorpora-

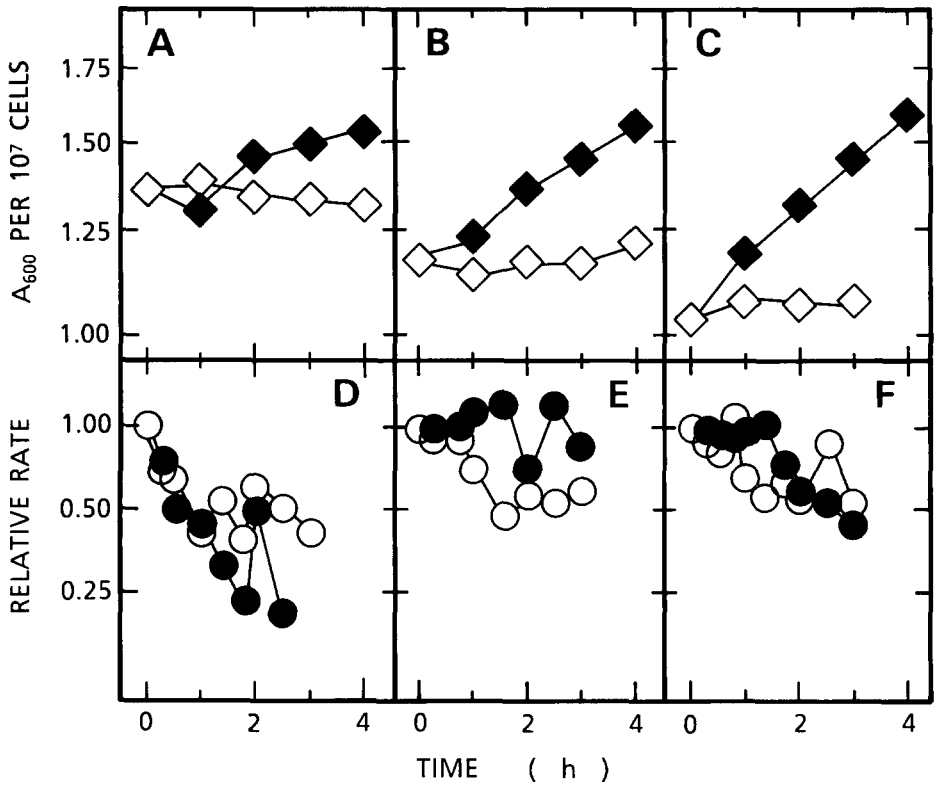

FIGURE 2.—Biosynthetic activity of mutant cells. *Turbidity*: cultures of proliferating *cdc65-1* (*panel A*), *cdc67-1* (*panel B*), and *cdc68-1* (*panel C*) mutant cells were divided, and one portion was transferred to 36° for further incubation. Cell concentration and turbidity (A_{600}) were determined for each fixed 1-ml sample. Symbols: ♦, cells at 36°; ◇, cells at 23°. *Label incorporation*: cultures of proliferating wild-type cells and *cdc65-1* (*panel D*), *cdc67-1* (*panel E*), and *cdc68-1* (*panel F*) mutant cells were transferred to 36°, and at intervals 1-ml samples were removed to prewarmed tubes containing both [^{14}C]uracil and [^{3}H]histidine. After further incubation at 36° for 10 min, incorporation of radiolabeled precursors was stopped by the addition of an equal volume of cold 10% trichloroacetic acid. Values for incorporation rate into acid-precipitable material by mutant cells were normalized, per cell, to the incorporation rates for wild-type cells treated in the same way. Symbols: ○, relative [^{14}C]uracil incorporation per mutant cell; ●, relative [^{3}H]histidine incorporation per mutant cell.

tion into protein and RNA, consistent with the observed turbidity increase.

Conjugation by arrested mutant cells: Some mutant cells that arrest at start and maintain biosynthetic activity also retain the ability to undergo conjugation in the arrested state (REED 1980). This criterion of mating competence was applied to mutant strains harboring the new start mutations described here.

A standard mating assay (BEDARD *et al.* 1984) was used to quantify the mating efficiencies of arrested mutant cells, as normalized to that of the related wild-type strain GR2; for comparison, previously characterized mating-proficient and mating-incompetent mutant strains were similarly tested (Table 4). The *cdc68-1* mutant strain remained competent to conjugate and in fact was as proficient in this mating assay as a *cdc28-4* mutant strain similarly tested (BEDARD *et al.* 1984). Strains bearing the *cdc65-1* or *cdc67-1* mutation, otherwise competent in mating at the permissive temperature of 23° (data not shown), lost mating ability under start-arrest conditions (Table 4). Furthermore, in mating mixtures containing these mutant cells an intermediate step in conjugation, that of zygote formation, was also impaired (Table 4). This finding distinguishes the mating deficiencies caused by the *cdc65-1* and *cdc67-1* start mutations from the karyogamy defects caused by certain other start mutations (DUTCHER and HARTWELL 1982), and suggests that the mating defects in these *cdc* mutant cells occur early in the pathway of response to mating pheromones.

TABLE 4

Conjugation by new start mutants

Mutation	Diploid formation (%)[a]	Zygote formation (%)[b]
+	100	25
cdc62-1	22	ND
cdc64-1	0.15	ND
cdc65-1	0.02	0.7
cdc67-1	0.1	0.9
cdc68-1	9.2	13.2

Matings were performed as described in MATERIALS AND METHODS. All strains were equally competent in diploid formation at 23° (data not shown).

[a] The efficiency of diploid formation by wild-type cells treated by the same temperature regime was set to 100%. No loss of viability occurred for any strain during the time course of the experiment (data not shown).

[b] Zygotes at the time of plating for diploids (approximately 400 cells scored per observation). Zygote and mating data derive from separate experiments. ND, not determined.

Cloning the new *CDC* genes: Yeast genomic sequences that complemented the temperature-sensitive defects in *cdc65-1*, *cdc67-1* and *cdc68-1* mutant strains were isolated from a yeast genomic DNA library (Figure 3). For two of the three mutations, independent temperature-resistant transformants were found by restriction mapping to contain recombinant plasmids with overlapping yeast genomic inserts, suggesting that in each of these *cdc* mutant recipient strains the same complementing sequence had been isolated repeatedly; one member of each group of related plasmids complementing a *cdc* mutation was chosen for

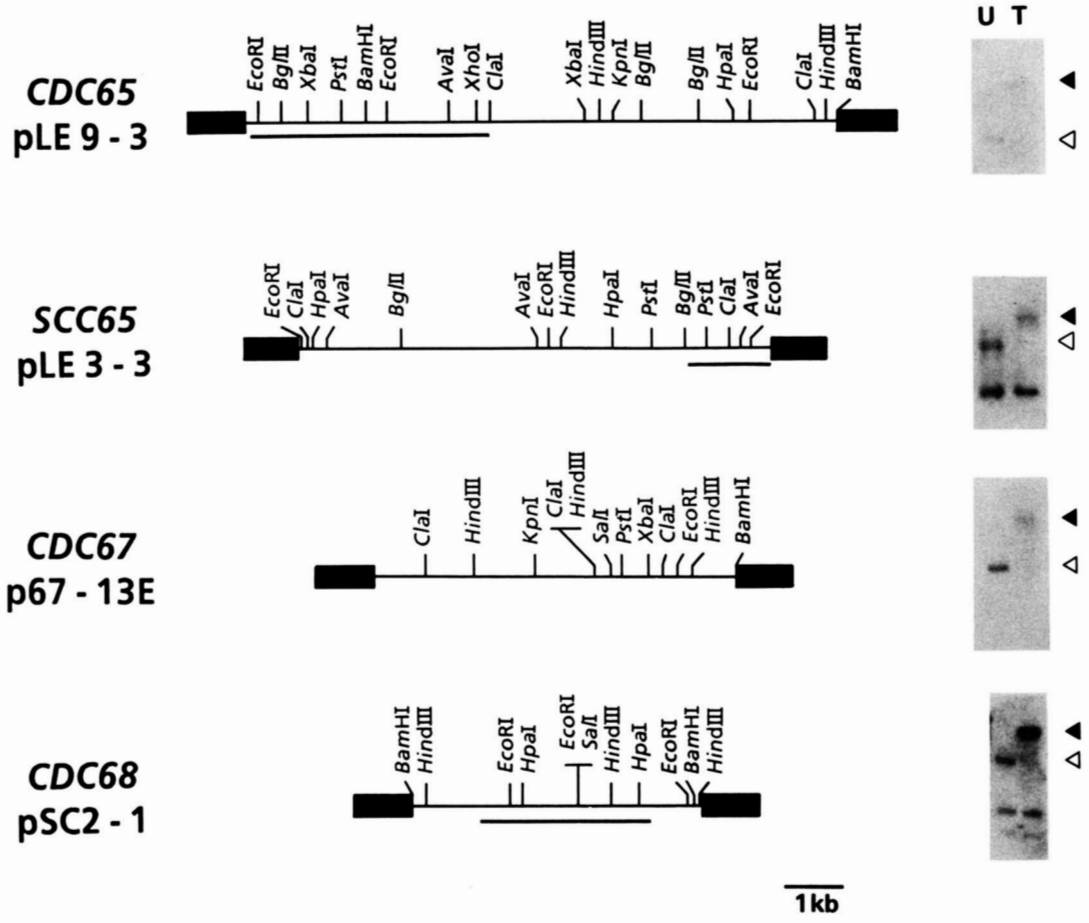

FIGURE 3.—Clones relieving the temperature sensitivity of new start mutations. Genomic insert sequences are indicated by thin lines, and flanking vector sequences by thick lines. Subcloned complementing insert regions are underlined. The two different sequences that relieve the temperature sensitivity of *cdc65-1* are both shown; in the other cases only one of the overlapping inserts is shown. To the right of each restriction map is a Southern analysis (see MATERIALS AND METHODS) of genomic DNA isolated from untransformed (U) and transformed (T) cells, confirming directed integration (◀) of each cloned sequence at the homologous chromosomal locus (◁). For the *CDC65* integrant, *Xho*I-digested DNA was probed with a 6-kb *Eco*RI fragment from the integration plasmid that contained insert and vector sequences but not the *URA3* gene. For the *SCC65* integrant, DNA digested with *Sst*I and *Xho*I was probed with the integration plasmid, including the *URA3* gene. For the *CDC67* integrant, *Bgl*II-digested DNA was probed with the same 2-kb *Hin*dIII fragment used to construct the integration vector (Figure 3; see MATERIALS AND METHODS). For the *CDC68* integrant, Southern blots of *Kpn*I-digested DNA were probed with the integration plasmid. The restriction fragment containing the *ura3-52* locus is thus also shown in the blots for *SCC65* and *CDC68*.

further study. In each case, loss of the plasmid vector marker *URA3* resulted in concomitant loss of temperature resistance, while retransformation of the *cdc* mutant strain with the purified plasmid restored temperature resistance, thus confirming complementation of the *cdc* mutation by the genomic insert.

Plasmid derivatives were integrated (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981) into the yeast genome at the site of insert homology (see MATERIALS AND METHODS), and confirmed by Southern analysis (Figure 3). Genetic analysis of mutant strains containing these integrated plasmids showed tight linkage of the vector marker *URA3* to the temperature-sensitive mutation (see MATERIALS AND METHODS), indicating that the plasmid had integrated by homologous recombination at the *cdc* locus and providing strong evidence that the genomic insert contained the *CDC* gene.

Transformation of the *cdc65-1* mutant strain yielded two different genomic sequences that made mutant cells temperature-resistant upon retransformation. This finding suggested that at least one of these inserts contains a heterologous sequence that suppresses the *cdc65-1* defect, rather than supplying wild-type *CDC65* function. Plasmid-integration analysis (Figure 3) showed that the *cdc65-1* mutation is tightly linked to the genomic sequence cloned within plasmid pLE9-3 (Figure 3), suggesting that this plasmid contains the wild-type *CDC65* gene. Integration of the other cloned sequence by homologous recombination within the insert (Figure 3) did not yield genetic linkage between *cdc65-1* and the integrated vector marker (see MATERIALS AND METHODS), showing that the genomic insert in plasmid pLE3-3 contains an unlinked suppressor sequence. Furthermore, the suppressor sequence subcloned into the centromere-containing shuttle vector YCp50 (Rose 1987) did not

TABLE 5

Genetic linkage

Genetic interval	Ascus type (No.) PD	NPD	TT	Map distance (cM)[a]
cdc65–ade4	15	0	26	31.7
cdc65–rna1	22	0	23	25.6
cdc67–tsm4572	16	2	28	46.2
cdc67–aro7	42	0	24	18.2
aro7–tsm4572	28	1	17	25.0
cdc68–ade5	44	0	20	15.6
cdc68–KEX1::LEU2	59	0	5	3.9
ade5–KEX1::LEU2	41	0	23	18.0

[a] Genetic distances were calculated as specified by MORTIMER and SCHILD (1985).

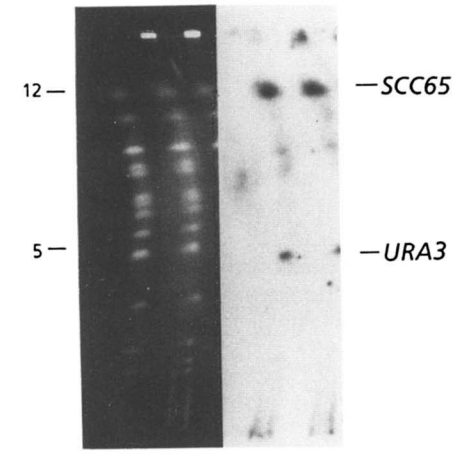

FIGURE 4.—Chromosomal assignment of *SCC65* sequences. Chromosomes were separated by CHEF electrophoresis (CHU, VOLLRATH and DAVIS 1986) (*left panel*), transferred to a nylon membrane and probed with plasmid pLE3-3 containing the *SCC65* and *URA3* genes (*right panel*). The identities of chromosomes probed by *SCC65* and *URA3* are indicated on the right side of the figure.

suppress the temperature sensitivity of the transformed *cdc65-1* mutant (data not shown), showing that suppression is a function of increased gene dosage, and perhaps of overexpression. The suppressor gene within the cloned genomic insert of plasmid pLE3-3 has been named *SCC65*.

Transformation of a *cdc65-33* (*dna33*) mutant strain with the episomal plasmid pLE9-3 produced temperature-resistant transformants that concomitantly lost temperature resistance when the *URA3* marker was lost. These transformation results are consistent with the allelic nature of the two *cdc65* mutations. More interestingly, transformation of the same *cdc65-33* strain with the episomal suppressor plasmid pLE3-3 also gave rise to temperature-resistant transformants that showed concomitant loss of the *URA3* vector marker and of temperature resistance. Suppression by the *SCC65* sequences on plasmid pLE3-3 is thus not allele-specific.

Genetic mapping: The genetic map position of the *cdc65-1* mutation was previously shown by a chromosome-loss procedure to be on the right arm of chromosome *XIII*, between *rna1* and *ade4* (HANIC-JOYCE 1985; Table 5). In these earlier mapping studies the *cdc65-1* mutation was erroneously designated *cdc61-1*. The *cdc61* designation has been removed from the yeast genetic map (D. SCHILD, personal communication).

To determine the genetic map positions of the remaining new *cdc* mutations and of the *cdc65* suppressor sequence *SCC65*, the cloned sequences were used to identify the yeast chromosome of origin. Each recombinant plasmid hybridized to two different chromosomes that had been resolved electrophoretically: one of these was chromosome *V*, probed by the *URA3* sequences on the plasmid vector, and the other was the chromosome bearing the sequence of interest (Figure 4, and data not shown). For example, Figure 4 shows that the *SCC65* suppressor sequence is derived from chromosome *XII*. Similar blots localized *CDC67* on chromosome *XVI* and *CDC68* on chromosome *VII* (data not shown). Tetrad analysis then positioned the mutation marking each of the *CDC* genes (Table 5). As suggested by the complementation results above, none of the *cdc* mutations mapped to a previously known gene (MORTIMER and SCHILD 1985).

DISCUSSION

Using a novel selection procedure based on cell enlargement, we have identified new mutations that arrest yeast cell proliferation in uniform fashion. Three of these mutations are of particular interest here. These mutations differentially block the performance of start, and define several previously unidentified genes.

The procedure used here was based on observations that many *cdc* mutants, including some start mutants, continue biosynthetic activity when blocked in proliferation (JOHNSTON, PRINGLE and HARTWELL 1977). Thus it was expected that some of the temperature-sensitive mutants identified through the enrichment procedure for enlarged cells described here would express a *cdc* phenotype, and furthermore that many of these *cdc* mutants would be blocked at start without significant impairment in biosynthetic activity. These expectations were realized (Table 2; Figure 2).

The previous isolations of start mutations have in many cases been facilitated by different enrichment procedures that exploit various properties of start mutant strains (see WHEALS 1987). For example, the ability of *cdc28* mutant cells to undergo conjugation upon arrest at start dictated the scheme that was used to isolate start mutations in the *CDC36*, *CDC37* and *CDC39* genes, as well as more *cdc28* mutations (REED 1980). By a different selection scheme, new mutations in the *CYR1* (*CDC35*) gene and in four other genes

were identified based on the biosynthetic defects they produced (BOUTELET, PETITJEAN and HILGER 1985), and the new start mutation that identified the *CDC60* gene was similarly obtained (BEDARD, JOHNSTON and SINGER 1981). Yet another selection scheme designed to isolate mutant cells blocked at start without regard for growth abilities led to the identification of the *cdc62-1*, *cdc64-1* and *cdc63-1* (*prt1-63*; HANIC-JOYCE, SINGER and JOHNSTON 1987) start mutations (BEDARD, JOHNSTON and SINGER 1981). The gradient enrichment scheme described here thus augments procedures available for mutant isolation by making use of another characteristic of *cdc* mutants, that of cell enlargement.

Two of the mutations described here prevented mutant cells from forming zygotes efficiently at the nonpermissive temperature (Table 4), despite the maintenance of significant (though decreased) levels of biosynthetic activity (Figure 2). In comparison, the *prt1-63* start mutation also decreases protein and RNA synthesis to levels similar to those in the *cdc65-1* and *cdc67-1* mutant cells (BEDARD, JOHNSTON and SINGER 1981), but start-arrested *prt1-63* mutant cells mate well (BEDARD *et al.* 1984). Thus the degree of impairment of global biosynthetic activity in *cdc65-1* and *cdc67-1* mutant cells seems insufficient to account for the impaired zygote formation. By analogy with other genes that encode proteins necessary for start and for karyogamy during conjugation (DUTCHER and HARTWELL 1982), perhaps the *CDC65* and *CDC67* genes encode gene products required for cell fusion as well as for start.

A striking finding from this study is the underrepresentation of previously identified genes that affect start. In particular, mutations in the *CDC28*, *CDC36*, *CDC37* or *CDC39* genes were not obtained, even though the basic tenet (conjugational proficiency) of the selection scheme used by Reed (1980) to obtain start mutations in those genes is contingent on the criterion used here (continued biosynthetic activity leading to cell enlargement). This finding that related selection schemes identify nonoverlapping sets of start mutations suggests that the catalog of genes that affect start relatively directly may be larger than expected.

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LITERATURE CITED

BEDARD, D. P., G. C. JOHNSTON and R. A. SINGER, 1981 New mutations in the yeast *Saccharomyces cerevisiae* affecting completion of "start." Curr. Genet. **4:** 205–214.

BEDARD, D. P., A. W. LI, R. A. SINGER and G. C. JOHNSTON, 1984 Mating ability during chemically induced G1 arrest of cells of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **160:** 1196–1198.

BOUTELET, F., A. PETITJEAN and F. HILGER, 1985 Yeast *cdc35* mutants are defective in adenylate cyclase and are allelic with *cyr1* mutants while *CAS1*, a new gene, is involved in the regulation of adenylate cyclase. *EMBO J.* **4:** 2635–2641.

BÜCKING-THROM, E., W. DUNTZE, L. H. HARTWELL and T. R. MANNEY, 1973 Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusable sex factor. Exp. Cell Res. **76:** 99–110.

CHU, G., D. VOLLRATH and R. W. DAVIS, 1986 Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234:** 1582–1585.

DMOCHOWSKA, A., D. DIGNARD, D. HENNING, D. Y. THOMAS and H. BUSSEY, 1987 Yeast *KEX1* gene encodes a putative protease with a carboxypeptidase B-like function involved in killer toxin and α-factor precursor processing. Cell **50:** 573–584.

DUMAS, L. B., J. P. LUSSKY, E. J. MCFARLAND and J. SHAMPAY, 1982 New temperature-sensitive mutants of *Saccharomyces cerevisiae* affecting DNA replication. Mol. Gen. Genet. **187:** 42–46.

DUTCHER, S. K., and L. H. HARTWELL, 1982 The role of *S. cerevisiae* cell division cycle genes in nuclear fusion. Genetics **100:** 175–184.

FINK, G. R., 1970 The biochemical genetics of yeast. Methods Enzymol. **17A:** 59–78.

HADWIGER, J. A., C. WITTENBERG, M. D. MENDENHALL and S. I. REED, 1989 The *Saccharomyces cerevisiae CKS1* gene, a homolog of the *Schizosaccharomyces pombe suc1*$^+$ gene, encodes a subunit of the Cdc28 protein kinase complex. Mol. Cell. Biol. **9:** 2034–2041.

HANIC-JOYCE, P. J., 1985 Mapping *cdc* mutations in the yeast *S. cerevisiae* by *rad52*-mediated chromosome loss. Genetics **110:** 591–607.

HANIC-JOYCE, P. J., G. C. JOHNSTON and R. A. SINGER, 1987 Regulated arrest of cell proliferation mediated by yeast *prt1* mutations. Exp. Cell Res. **172:** 134–145.

HANIC-JOYCE, P. J., R. A. SINGER and G. C. JOHNSTON, 1987 Molecular characterization of the yeast *PRT1* gene in which mutations affect translation initiation and regulation of cell proliferation. J. Biol. Chem. **262:** 2845–2851.

HARTWELL, L. H., 1967 Macromolecule synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. **93:** 1662–1670.

HARTWELL, L. H., 1970 Periodic density fluctuation during the yeast cell cycle and the selection of synchronous cultures. J. Bacteriol. **104:** 1280–1285.

HARTWELL, L. H., 1974 *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. **38:** 164–198.

HARTWELL, L. H., J. CULOTTI and B. REID, 1970 Genetic control of the cell-division cycle in yeast. I. Detection of mutants. Proc. Natl. Acad. Sci. USA **66:** 352–359.

HARTWELL, L. H., C. S. MCLAUGHLIN and J. R. WARNER, 1970 Identification of ten genes that control ribosome formation in yeast. Mol. Gen. Genet. **109:** 42–56.

HARTWELL, L. H., R. K. MORTIMER, J. CULOTTI and M. CULOTTI, 1973 Genetic control of the cell division cycle in yeast. V. Genetic analysis of *cdc* mutants. Genetics **74:** 267–286.

HEREFORD, L. M., and L. H. HARTWELL, 1974 Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. J. Mol. Biol. **84:** 445–461.

HINNEN, A., J. B. HICKS and G. R. FINK, 1978 Transformation of yeast. Proc. Natl. Acad. Sci. USA **75:** 1929–1933

IIDA, H., and I. YAHARA, 1984 Specific early-G1 blocks accompanied with stringent response in *Saccharomyces cerevisiae* lead to growth arrest in resting state similar to the GO of higher eucaryotes. J. Cell Biol. **98:** 1185–1193.

JAHNG, K., J. FERGUSON and S. I. REED, 1988 Mutations in a gene encoding the α subunit of a *Saccharomyces cerevisiae* G protein indicate a role in mating pheromone signaling. Mol. Cell. Biol. **8:** 2484–2493.

JOHNSTON, G. C., J. R. PRINGLE and L. H. HARTWELL, 1977 Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. Exp. Cell Res. **105:** 79–98.

JOHNSTON, G. C., and R. A. SINGER, 1978 RNA synthesis and control of cell division in the yeast *S. cerevisiae*. Cell **14:** 951–958.

JOHNSTON, L. H., and J. C. GAME, 1978 Mutants of yeast with depressed DNA synthesis. Mol. Gen. Genet. **161:** 205–214.

JOHNSTON, L. H., and A. P. THOMAS, 1982 The isolation of new DNA synthesis mutants in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **186:** 439–444.

JOHNSTON, S. A., and J. E. HOPPER, 1982 Isolation of the yeast regulatory gene *GAL4* and analysis of its dosage effects on the galactose/melibiose regulon. Proc. Natl. Acad. Sci. USA **79:** 6971–6975.

LORINCZ, A. T., and S. I. REED, 1984 Primary structure homology between the product of yeast cell division control gene *CDC28* and vertebrate oncogenes. Nature **307:** 183–185.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MILLER, J. H., 1972 *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MORTIMER, R. K., and D. C. HAWTHORNE, 1973 Genetic mapping in *Saccharomyces*. IV. Mapping of temperature-sensitive genes and use of disomic strains in localizing genes. Genetics **74:** 33–54.

MORTIMER, R., and D. SCHILD, 1981 Genetic mapping in *Saccharomyces cerevisiae*, pp. 11–26 in *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance*, edited by J. N. STRATHERN, E. W. JONES and J. R. BROACH. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MORTIMER, R., and D. SCHILD, 1985 Genetic map of *Saccharomyces cerevisiae*, edition 9. Microbiol. Rev. **49:** 181–212.

ORR-WEAVER, T. L., J. W. SZOSTAK and R. L. ROTHSTEIN, 1981 Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA **78:** 6354–6358.

PRINGLE, J. R., and L. H. HARTWELL, 1981 The *Saccharomyces cerevisiae* cell cycle, pp. 98–142 in *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance*, edited by J. N. STRATHERN, E. W. JONES and J. R. BROACH. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

REED, S. I., 1980 The selection of *S. cerevisiae* mutants defective in the start event of cell division. Genetics **95:** 561–577.

REED, K. C., and D. A. MANN, 1985 Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13:** 7207–7221.

REED, S. I., J. A. HADWIGER, M. D. MENDENHALL, H. E. RICHARDSON and C. WITTENBERG, 1988 Control of cell proliferation in *Saccharomyces cerevisiae* by the Cdc28 protein kinase, pp. 53–56 in *Cell Cycle Control in Eukaryotes*, edited by D. BEACH, C. BASILICO and J. NEWPORT. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

ROBINSON, L. C., J. B. GIBBS, M. S. MARSHALL, I. S. SIGAL and K. TATCHELL, 1987 *CDC25*: a component of the *RAS*-adenylate cyclase pathway in *Saccharomyces cerevisiae*. Science **235:** 1218–1221.

ROSE, M., 1987 Isolation of genes by complementation in yeast. Methods Enzymol. **152:** 481–504.

ROTHSTEIN, R. J., 1986 Cloning in yeast, pp. 45–66 in *DNA Cloning*, Vol. 2, edited by D. M. GLOVER. IRL Press, Oxford.

SHULMAN, R. W., L. H. HARTWELL and J. R. WARNER, 1973 Synthesis of ribosomal proteins during the yeast cell cycle. J. Mol. Biol. **73:** 513–525.

STORMS, R. K., R. W. ORD, M. T. GREENWOOD, B. MIRDAMADI, F. K. CHU and M. BELFORT, 1984 Cell cycle-dependent expression of thymidylate synthase in *Saccharomyces cerevisiae*. Mol. Cell Biol. **4:** 2858–2864.

WHEALS, A. E., 1987 Biology of the cell cycle in yeasts, pp. 283–390 in *The Yeasts*, edited by A. H. ROSE and J. S. HARRISON. Academic Press, London.

WITTENBERG, C., and S. I. REED, 1988 Control of the yeast cell cycle is associated with assembly/disassembly of the Cdc28 protein kinase complex. Cell **54:** 1061–1072.

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