MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 1034–1041
0270-7306/93/021034-08$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 13, No. 2

Heat Shock-Mediated Cell Cycle Blockage and G_1 Cyclin Expression in the Yeast *Saccharomyces cerevisiae*

ADELE ROWLEY,[1] GERALD C. JOHNSTON,[1]* BRAEDEN BUTLER,[2] MARGARET WERNER-WASHBURNE,[2] AND RICHARD A. SINGER[3,4]

Departments of Microbiology and Immunology,[1] Biochemistry,[3] and Medicine,[4] Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7, and Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131[2]

Received 27 May 1992/Returned for modification 16 July 1992/Accepted 6 November 1992

For cells of the yeast *Saccharomyces cerevisiae*, heat shock causes a transient inhibition of the cell cycle-regulatory step START. We have determined that this heat-induced START inhibition is accompanied by decreased *CLN1* and *CLN2* transcript abundance and by possible posttranscriptional changes to *CLN3* (*WHI1*/*DAF1*) cyclin activity. Persistent *CLN2* expression from a heterologous promoter or the *CLN2-1* or *CLN3-1* alleles that are thought to encode cyclin proteins with increased stability eliminated heat-induced START inhibition but did not affect other aspects of the heat shock response. Heat-induced START inhibition was shown to be independent of functions that regulate cyclin activity under other conditions and of transcriptional regulation of *SWI4*, an activator of cyclin transcription. Cells lacking Bcy1 function and thus without cyclic AMP control of A kinase activity were inhibited for START by heat shock as long as A kinase activity was attenuated by mutation. We suggest that heat shock mediates START blockage through effects on the G_1 cyclins.

Cell proliferation by the budding yeast *Saccharomyces cerevisiae* is regulated primarily at a central control step in G_1 named START. Performance of START requires activation of a highly conserved protein kinase that, for *S. cerevisiae*, is encoded by the *CDC28* gene (28). The product of the *CDC28* gene, termed p34 kinase, is activated when complexed with other proteins termed G_1 cyclins, the products of a functionally redundant family of genes, *CLN1*, *CLN2*, and *CLN3* (*WHI1*/*DAF1*) (17, 31, 36, 53). The absence of *CLN* gene expression leads to the arrest of cell proliferation at START as a consequence of failure to activate the p34 protein kinase (7, 37).

START is also affected by an abrupt transfer of proliferating wild-type cells to an elevated growth temperature. This thermal shock induces a variety of cellular responses, referred to collectively as the heat shock response (27). Among these responses is a transient inhibition of START (24, 42) that causes heat-shocked wild-type populations to accumulate transiently as unbudded cells (24). These cells then spontaneously recover, even under heat shock conditions, so that START is performed and the cells resume proliferation. We have demonstrated that heat shock blocks START without removing cells from the mitotic cell cycle (11).

In the course of other studies, we noticed that heat shock results in decreased transcript abundance for a number of genes, including the *CLN1* and *CLN2* genes that encode two of the G_1 cyclins described above (38). We have now more thoroughly investigated these transcriptional effects and show here that the characteristic inhibition of START by heat shock is affected by altered cyclin expression. We have also assessed the effect of altered cyclin protein stability (17, 31, 36) on the heat shock response and the involvement of known regulators of G_1 cyclin expression on heat-induced cell cycle blockage.

* Corresponding author.

MATERIALS AND METHODS

Strains and plasmids. The yeast strains used are listed in Table 1. Plasmids YCpG2-CLN2 (53), containing the wild-type *CLN2* gene under the control of the *GAL1* promoter, and Bd824, containing the *SWI4* gene under *GAL* control, were provided by C. Wittenberg and L. Breeden, respectively. The *CLN3* (*WHI1*/*DAF1*) gene was disrupted by using plasmid pWJ310 (31), provided by B. Futcher, and the disruption was confirmed by Southern analysis. For each analysis, the behavior of mutant strains was compared with that of an appropriate wild-type control strain.

Culture conditions and assessment of cellular parameters. Cells were grown in YM1 complex liquid medium supplemented with 2% glucose or in YNB defined liquid medium supplemented with 2% glucose, amino acids (40 μg/ml), and nucleotide bases (20 μg/ml) as required to satisfy auxotrophies (18, 22). Cell concentration was determined with an electronic particle counter (Coulter Electronics Inc.), and cell morphology was assessed by direct microscopic inspection (19). Routinely, proliferating cells were subjected to heat shock at cell concentrations of approximately 5×10^6 cells per ml. Before assessment of cellular parameters, cells were fixed in Formalin and sonicated briefly to disrupt any clumps (19), and at least 200 cells were scored for each determination. Acquired thermotolerance was assessed as described before (2).

Northern (RNA blot) analysis. Cells were grown at 23°C to a concentration of 4×10^6 to 6×10^6 cells per ml before a portion of the culture was shifted to 37°C and incubated further. Total RNA was extracted as described before (35, 41). Equal amounts of RNA (usually 20 μg per lane) were denatured and resolved electrophoretically through formaldehyde-agarose gels (29). RNA was transferred to a nylon membrane (NEN Research Products) and cross-linked by UV irradiation with a model 2400 cross-linker (Stratagene). Hybridization with restriction fragments was done as described before (45).

TABLE 1. *S. cerevisiae* strains

Strain	Relevant genotype[a]	Source[b] (reference)
GR2	*his6 ura1*	DYL (22)
LDW6A	*his CLN3-1*	DYL (49)
21R	*ade1 leu2-3,112 ura3-52*	J. E. Hopper
JHY627	*ade1 his3 leu2-3,112 trp1 ura3*	C. Wittenberg
JHY629	*ade1 his3 leu2-3,112 trp1 ura3 cln1::URA3*	C. Wittenberg
JHY631	*ade1 his3 leu2-3,112 trp1 ura3 cln2::LEU2*	C. Wittenberg
JHY633	*ade1 his3 leu2-3,112 trp1 ura3 cln1::URA3 cln2::LEU2*	C. Wittenberg
GCY24	*ade1 his3 leu2-3,112 trp1 ura3 CLN2-1*[c]	C. Wittenberg
CWY231	*ade1 his3 leu2-3,112 trp1 ura3Δns*	C. Wittenberg
CWY229	*ade1 his3 leu2-3,112 trp1 ura3Δns cln2::LEU2*	C. Wittenberg
BF338-2a	*ade1 his3 ura3*	B. Futcher
BF338-2a whi::URA3	*ade1 his3 ura3 cln3::URA3*	B. Futcher
FC279	*Δura3 his2 ade1 trp1 leu2 bar1::LEU2*	F. Chang and I. Herskowitz (6)
FC280	*Δura3 his2 ade1 trp1 leu2 bar1::LEU2 far1::URA3*	F. Chang and I. Herskowitz (6)
L3999[d]	*ura3-52 trp1Δ1 leu2-3,112 lys2-801*	J. Brill and G. Fink (13)
L4645[d]	*ura3-52 trp1Δ1 leu2-3,112 lys2-801 fus3-6::URA3*	J. Brill and G. Fink (13)
RS13-7C-1	*his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2^{w1} tpk3::TRP1 bcy1::LEU2*	M. Wigler (33)
RS58Ac3	*his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 cln3::URA3*	This study

[a] All strains are *MAT***a**.
[b] DYL, Dalhousie Yeast Laboratory.
[c] The *CLN2-1* mutant gene is integrated at the *TRP1* locus.
[d] Strains L3999 and L4645 were previously named EEX171-13B and EY419 (13), respectively (3a).

Restriction fragments used to visualize transcripts. The *CLN1* probe was a 1.6-kbp *Nde*I-*Bam*HI fragment carried on plasmid pRK171, and the *CLN2* probe was a *Bam*HI fragment from pUC10-CLN2, both provided by C. Wittenberg. The *CLN3* probe was a 1.6-kbp *Eco*RI-*Xho*I fragment from a plasmid containing the *CLN3* gene, provided by F. Cross. The specificity of each cyclin probe was confirmed by Northern analysis of RNA from cyclin-disrupted strains (data not shown). The *ACT1* probe was a 1-kbp *Hin*dIII-*Xho*I fragment from pRS208, a gift from R. Storms. The *SWI4* probe, provided by L. Breeden, was a 3.1-kbp *Pst*I-*Bam*HI fragment from pIC19R, and the *SSA3* probe was a 750-bp *Rsa*I fragment from pUC9-SSA3.

RESULTS

Heat shock inhibits cyclin gene expression and START. Cells proliferating at 23°C were transferred to 37°C, and samples were removed at intervals for assessment of bud morphology. (For this yeast, the presence of a bud reflects the cell cycle position: cells in the G_1 interval of the cell cycle are unbudded [20].) As expected (24), wild-type cells responded to this heat shock by transiently accumulating in the unbudded (G_1) interval of the cell cycle, at the regulatory step START (Fig. 1A). The heat-induced inhibition of START is only temporary, and cells soon resume proliferation even when maintained at the elevated temperature (24) (data not shown). We determined the abundance of cyclin transcripts for these heat-shocked cells. As shown in Fig. 1B, *CLN1* and *CLN2* transcript abundance was decreased by 20 min after the transfer to 37°C. Like the effect of heat shock on START, the effect of heat shock on *CLN1* and *CLN2* transcript abundance was also transient. Within 40 to 60 min after the transfer to 37°C, the levels of *CLN1* and *CLN2* transcripts increased (Fig. 1B). Thus, upon heat shock the inhibition of cyclin expression occurs prior to the inhibition of START, and the recovery of cyclin transcript levels occurs prior to the performance of START. Because cyclin gene expression is necessary for the performance of START (37), it is reasonable to infer that heat shock could inhibit START, at least in part, by decreasing cyclin expression.

To test the effects of continued transcription of cyclin genes, we heat-shocked cells that were expressing the wild-type *CLN2* gene from a heterologous promoter, the *GAL1* promoter (37). As shown in Fig. 1G and H, for these cells growing in medium with galactose as the carbon source, the levels of *CLN2* transcript expressed from the *GAL1* promoter remained high during heat shock, and there were no signs of START inhibition (no accumulation of unbudded cells). This aberrant cell cycle response to heat shock was not simply the result of inhibition of progress through another stage of the cell cycle, because heat-shocked cells expressing high levels of *CLN2* transcript continued to proliferate, with kinetics indistinguishable from those of the isogenic wild-type control cells (data not shown). Persistent *CLN2* transcription during heat shock therefore suppresses the inhibition of START.

Expression of the *CLN2* gene from the inducible *GAL1* promoter results in greater transcript abundance than expression from the endogenous *CLN2* promoter (Fig. 1 legend). We therefore also determined the effect of lower levels of persistent *CLN2* expression on the heat shock response. Low concentrations of glucose have been shown to decrease but not abolish expression from *GAL* promoters (1), and we found that addition of 0.25% glucose to cells proliferating in 2% galactose decreased *GAL1*-regulated *CLN2* transcripts to a level only slightly higher than that of the endogenous *CLN2* transcript (Fig. 1I, and data not shown). (This experiment was performed with a *GAL1-CLN2* transformant harboring a chromosomal *cln2* null mutation [Table 1], so that the only detectable *CLN2* transcript was *GAL1* regulated.) As shown in Fig. 1I, after heat shock the *CLN2* transcript persisted at the same level as the endogenous *CLN2* transcript in proliferating control cells. This persistent but low-level *GAL1-CLN2* expression also markedly decreased the cellular response to heat shock (Fig. 1G, and data not shown). Thus, persistent *CLN2* expression at physiological levels during heat shock prevents the inhibition of START.

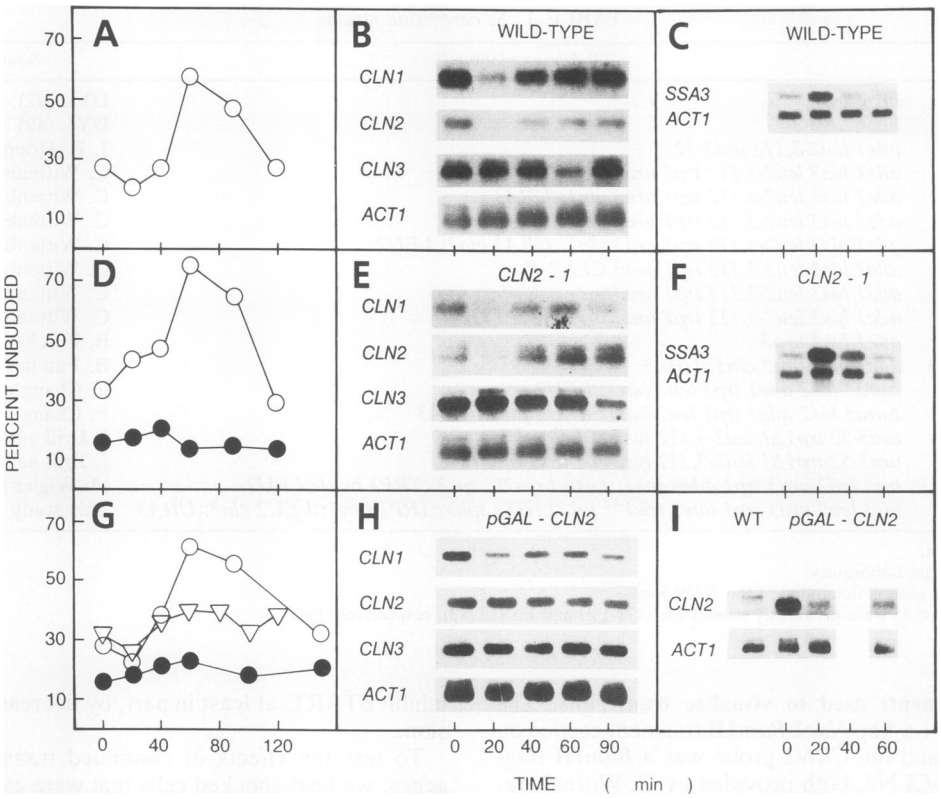

FIG. 1. Responses of START and transcription to heat shock. Wild-type (WT) cells of strain 21R (A, B, and C) and the same wild-type cells transformed with the pGAL-CLN2 plasmid (G and H) were transferred to 37°C at time zero and incubated for the times indicated. Likewise, cells of the wild-type strain JHY627 and the *CLN2-1* mutant strain GCY24 (D, E, and F), and the Δ*cln2* strain CWY229 transformed with the pGAL-CLN2 plasmid (G and I) were grown at 23°C and transferred to 37°C at time zero. Strain CWY229 was grown in defined medium lacking uracil to maintain the pGAL-CLN2 plasmid and supplemented with 2% galactose to express the *GAL*-regulated *CLN2* gene. Before heat shock, glucose was added to a final concentration of 0.25%, and cells were allowed to proliferate for at least three generations. Note that the *CLN2* transcript levels displayed in panel H for cells harboring the pGAL-CLN2 plasmid growing in 2% galactose alone were higher than those in untransformed wild-type cells, which in this exposure were barely detectable (data not shown). (A, D, and G) Cell morphology. ○, wild type; ●, mutant or transformed; △, pGAL-CLN2-transformed Δ*cln2* cells; (B, E, and H) *CLN1*, *CLN2*, *CLN3*, and *ACT1* transcript levels; (C and F) *SSA3* and *ACT1* transcript levels; (I) *CLN2* and *ACT1* transcript levels.

Increased cyclin stability prevents heat-induced START inhibition. All three G_1 cyclins have been inferred or demonstrated to be unstable proteins (7, 36, 37, 48, 53). Decreased cyclin transcript abundance might therefore be expected to cause a rapid depletion of cyclin proteins (37). Thus, the inhibition of cyclin gene transcription that we observe after heat shock may affect START in part by depleting the supply of functional Cln1 and Cln2 cyclins. Conversely, cyclin proteins that persist because of increased stability might prevent the heat shock-induced START inhibition. To test this idea, we took advantage of the *CLN2-1* allele, thought to encode a hyperactive and/or hyperstable form of the Cln2 cyclin protein (17, 36). The mutant cyclin protein encoded by *CLN2-1* lacks the C-terminal portion of the Cln2 protein, implicated in targeted cyclin degradation (17, 36).

The *CLN2-1* allele prevented the typical transient accumulation of unbudded cells after heat shock (Fig. 1D). As for heat-shocked cells expressing the *CLN2* gene from the heterologous *GAL* promoter, the failure of *CLN2-1* mutant cells to accumulate as unbudded cells was not simply the result of blockage elsewhere in the cell cycle; heat-shocked mutant cells continued to proliferate, with kinetics indistinguishable from those of the isogenic wild-type control cells (data not shown). Furthermore, the ability to see an accumulation of unbudded cells after heat shock was not simply obscured by the unusually low proportion of unbudded cells in *CLN2-1* mutant populations (17); heat-shocked wild-type cells proliferating in medium containing low concentrations of the S-phase inhibitor hydroxyurea to decrease the proportion of unbudded cells in the starting population (43) still displayed a dramatic and transient accumulation of unbudded cells (data not shown). Thus, the *CLN2-1* allele prevents the transient START inhibition after heat shock.

The lack of START inhibition in heat-shocked *CLN2-1* mutant cells was not reflected by *CLN* transcript levels; mutant cells still displayed the usual transient decrease in abundance of *CLN1* and *CLN2* (*CLN2-1*) transcripts after heat shock (Fig. 1E). Thus, the ability of the *CLN2-1* allele to prevent the heat-induced inhibition of START most likely resulted from the persistence of Cln2 activity rather than from continued Cln2 synthesis.

***CLN2-1* mutant cells display other heat shock responses.** The inability of *CLN2-1* mutant cells to respond to heat shock by transiently inhibiting START does not reflect a more general inability of mutant cells to respond to the stress imposed by heat shock. As another measure of the heat shock response, cells were assayed for the heat-inducible

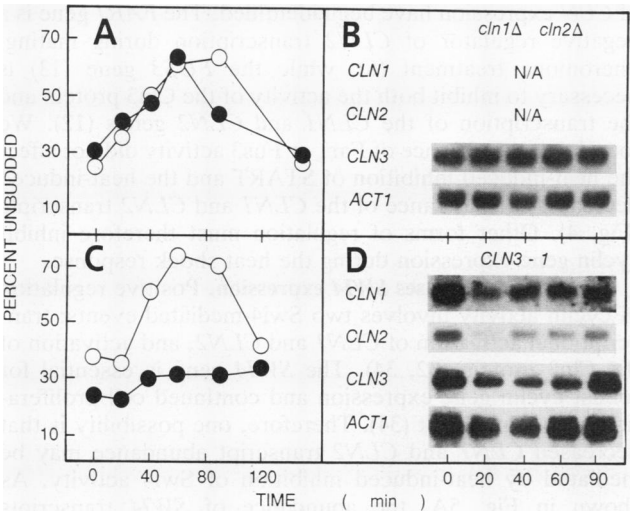

FIG. 2. Cln3 inactivation is necessary for heat-induced START inhibition. Cells of wild-type strain JHY627 and *cln1*::*URA3* *cln2*::*LEU2* double-disrupted strain JHY633 (A and B), and wild-type strain GR2 and *CLN3-1* mutant strain LDW6A (C and D) were heat shocked as described in the legend to Fig. 1. (A and C) Cell morphology. ○, wild type; ●, mutant. (B and D) Transcript levels. N/A, not applicable.

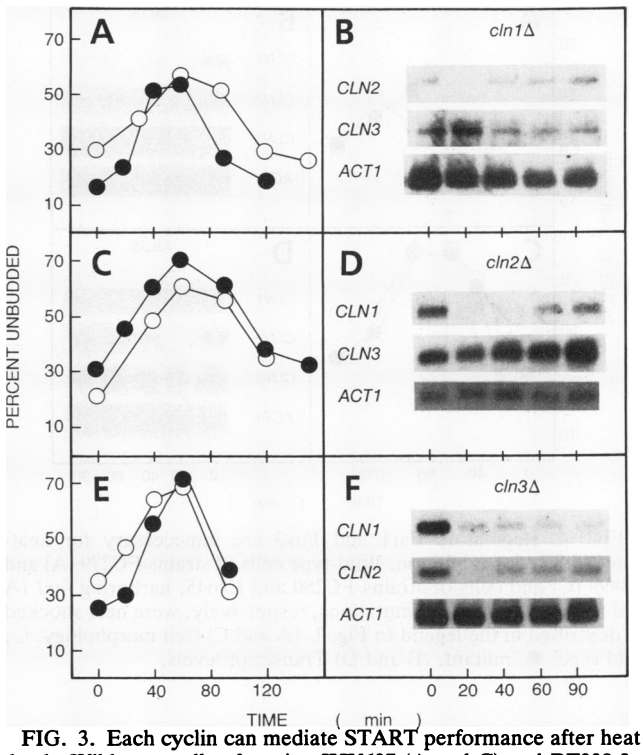

FIG. 3. Each cyclin can mediate START performance after heat shock. Wild-type cells of strains JHY627 (A and C) and BF338-2a (E) and cells of strains JHY629, JHY631, and BF338-2a whi::URA3, harboring the *cln1*::*URA3* (A and B), *cln2*::*LEU2* (C and D), or *cln3*::*URA3* (E and F) disruption, respectively, were heat shocked as described in the legend to Fig. 1. (A, C, and E) Cell morphology. ○, wild type; ●, mutant. (B, D, and F) Transcript levels.

SSA3 gene transcript levels (51, 52). As shown in Fig. 1F, *CLN2-1* mutant cells displayed a pattern of *SSA3* induction like that of wild-type cells. Heat-shocked cells are also able to survive a subsequent exposure to otherwise lethal temperatures, a property referred to as acquired thermotolerance (21, 30); transfer of wild-type cells to a temperature of 37°C (heat shock) allows these cells to then survive brief incubation at temperatures as high as 52°C. When *CLN2-1* mutant cells were incubated at 37°C and then transferred to 52°C, they displayed the same degree of acquired thermotolerance as did heat-shocked congenic wild-type cells (data not shown). Thus, *CLN2-1* mutant cells are suppressed only for certain aspects of the heat shock response, including heat-induced START inhibition.

Heat shock may inhibit Cln3 cyclin activity posttranscriptionally. Genetic studies have shown that any one of the three G_1 cyclin proteins supports the performance of START. This functional redundancy among members of the cyclin gene family implies that the transcriptional inhibition of *CLN1* and *CLN2* expression by heat shock might not be sufficient for the START inhibition that is seen and that the activity encoded by the *CLN3* gene may also have to be inhibited. We noted that heat shock had little effect on *CLN3* transcript levels (Fig. 1B), suggesting that if Cln3 protein activity is involved, it would have to be inhibited at a posttranscriptional level.

To examine the possibility that heat shock inhibits the activity encoded by the *CLN3* gene, we characterized the effects of heat shock on cells lacking both *CLN1* and *CLN2* (see Materials and Methods). These Δ*cln1* Δ*cln2* double-mutant cells are kept alive (and able to perform START) by the activity of the *CLN3* gene (37). We found that these Δ*cln1* Δ*cln2* cells also became transiently blocked at START after heat shock (Fig. 2A). In these double-mutant cells, as in wild-type cells, *CLN3* transcript abundance was not decreased by heat shock (Fig. 2B). The inhibition of START in Δ*cln1* Δ*cln2* cells, for which the *CLN3* gene is essential, suggests that Cln3 activity may be inhibited, but the maintenance of normal *CLN3* transcript levels in these mutant cells shows that any such inhibition does not occur at the transcriptional level.

To determine whether a hyperstable Cln3 protein could eliminate heat-induced START inhibition, we assessed the heat shock response of cells harboring the *CLN3-1* mutant allele (*WHI1-1* [44]), which encodes a truncated, hyperstable Cln3 protein (31, 48) and in this sense is analogous to the *CLN2-1* allele described above. As found for *CLN2-1*, the *CLN3-1* allele had no effect on the usual transient decrease in *CLN1* and *CLN2* transcript levels after heat shock (Fig. 2D) but prevented the transient inhibition of START (accumulation of unbudded cells) (Fig. 2C). *CLN3-1* mutant cells also continued through the cell cycle after the temperature shift, with kinetics similar to those of the wild-type control population (data not shown). The prevention of heat-induced START inhibition by the *CLN3-1* allele is consistent with a requirement for decreased Cln3 protein activity, mediated posttranscriptionally, to bring about START inhibition by heat shock.

Cyclin proteins are individually dispensable for heat-induced START inhibition and decreased transcription. In other situations, the transcription of the *CLN1* and *CLN2* genes has been found to be influenced by the activity of the Cln3 protein (8, 10), raising the possibility that the heat shock effects on *CLN1* and *CLN2* transcript levels could be mediated by an inhibition of Cln3 protein activity. However, we found that Δ*cln3* mutant cells, without Cln3 activity, also exhibited decreased *CLN1* and *CLN2* transcript levels after heat shock (Fig. 3, and data not shown). Furthermore,

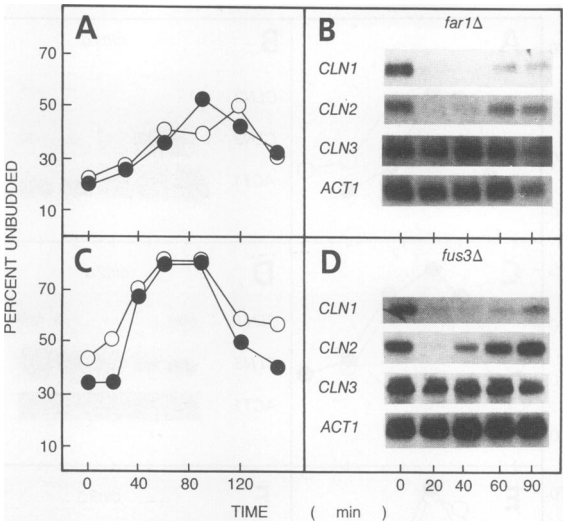

FIG. 4. Regulators Far1 and Fus3 are unnecessary for heat-induced START inhibition. Wild-type cells of strains FC279 (A) and L3999 (C) and cells of strains FC280 and L4645, harboring *far1* (A and B) or *fus3* (C and D) mutations, respectively, were heat shocked as described in the legend to Fig. 1. (A and C) Cell morphology. ○, wild type; ●, mutant. (B and D) Transcript levels.

single-mutant cells lacking Cln1 or Cln2 protein activity also showed normal transcriptional responses to heat shock (Fig. 3) despite the somewhat lower initial cyclin transcript levels in the Δ*cln1* mutant cells. These heat-induced decreases in transcript abundance for each single-mutant strain demonstrate that the transcriptional response to heat is not mediated by any single cyclin. Similarly, each single-mutant strain devoid of one of the G_1 cyclin proteins still showed the usual heat-induced inhibition of START (Fig. 3).

In some cases, including the Δ*cln3* experiment (Fig. 3F), we have noted prolonged decreases in *CLN1* transcript abundance. This variant *CLN1* response to heat shock was in each case also seen in closely related wild-type cells (data not shown). The delayed restoration of *CLN1* transcript abundance does not affect recovery from heat shock, since both wild-type and mutant cells proliferated after heat shock (data not shown).

Far1 and Fus3 regulatory proteins do not mediate heat-induced START inhibition. START inhibition and concurrent negative regulation of cyclin gene expression are also brought about by treatment of haploid yeast cells with mating pheromone. In that situation, two negative regulators of *CLN* expression have been identified. The *FAR1* gene is a negative regulator of *CLN2* transcription during mating-pheromone treatment (6), while the *FUS3* gene (13) is necessary to inhibit both the activity of the Cln3 protein and the transcription of the *CLN1* and *CLN2* genes (12). We found that the absence of Far1 or Fus3 activity did not affect the heat-induced inhibition of START and the heat-induced decreases in abundance of the *CLN1* and *CLN2* transcripts (Fig. 4). Other forms of regulation must therefore inhibit cyclin gene expression during the heat shock response.

Heat shock decreases *SWI4* expression. Positive regulation of cyclin activity involves two Swi4-mediated events: transcriptional activation of *CLN1* and *CLN2*, and activation of the Cln3 protein (32, 34). The *SWI4* gene is essential for robust cyclin gene expression and continued cell proliferation after heat shock (34). Therefore, one possibility is that decreased *CLN1* and *CLN2* transcript abundance may be mediated by heat-induced inhibition of Swi4 activity. As shown in Fig. 5A, the abundance of *SWI4* transcripts showed a modest transient decrease after heat shock, which paralleled the decreases in *CLN* transcript abundance. This *SWI4* transcript decrease, coupled with the short half-life of Swi4 protein (3), raises the possibility that heat shock may lead to decreased Swi4 levels.

To assess any involvement of Swi4 protein in heat-induced *CLN* transcriptional effects, strains were constructed in which *SWI4* gene expression was regulated by a *GAL* promoter and therefore largely unaffected by heat shock (3) (Fig. 5C). Cells transformed with a pGAL-SWI4 plasmid were grown on galactose to express *SWI4* from the heterologous *GAL* promoter. Heat-shocked cells carrying this pGAL-SWI4 construct still showed decreased *CLN1* and *CLN2* transcript abundance (Fig. 5C). The heat-induced effects on cyclin gene expression may therefore be modulated by a mechanism that is independent of *SWI4* transcriptional regulation, although we cannot exclude posttranscriptional modulation of Swi4 upon heat shock.

Heat-shocked cells expressing the *GAL-SWI4* gene also accumulated as unbudded cells (Fig. 5B), showing that this response to heat shock is also not abrogated by increased *SWI4* expression.

Heat-induced START inhibition does not require cAMP control of A kinase. Many features of the heat shock response, including acquired thermotolerance and accumulation of storage carbohydrates, are mediated by a cyclic AMP (cAMP)-mediated signal transduction pathway that modulates the activity of cAMP-dependent protein kinase (A kinase) (4). For *S. cerevisiae*, the *BCY1* gene encodes the regulatory subunit of A kinase (46); the absence of Bcy1

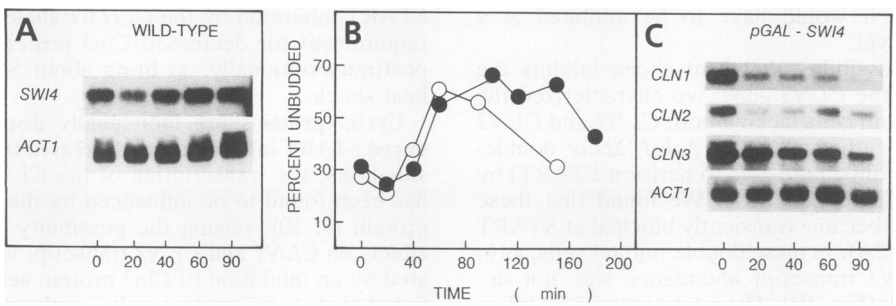

FIG. 5. *SWI4* transcript levels are decreased by heat shock. Wild-type cells of strain 21R with (C and B, ●) or without (A and B, ○) the pGAL-SWI4 plasmid were heat shocked as described in the legend to Fig. 1. (B) Cell morphology. (A and C) Transcript levels.

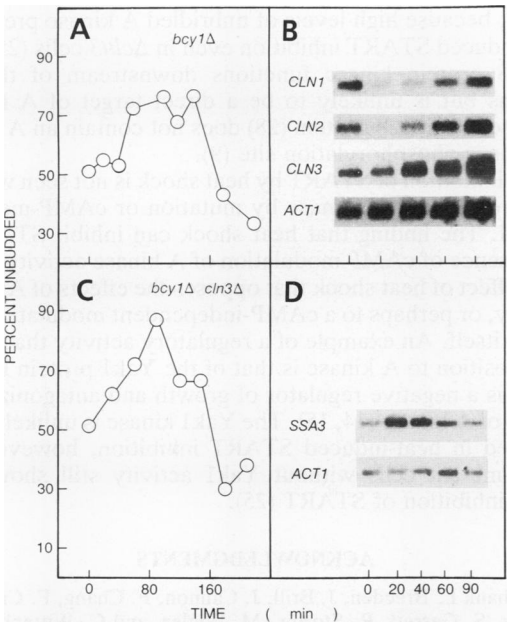

FIG. 6. Modulation of A kinase activity by cAMP is unnecessary for heat-induced START inhibition. Cells harboring the *bcy1*::*LEU2* disruption were heat shocked as described in the legend to Fig. 1. (A and C) Morphologies of cells of strains RS13-7C-1 and RS58Ac3, respectively; (B and D) transcript levels in cells of strain RS13-7C-1.

function leads to A kinase activity unbridled by cAMP (33). A mutation in the *BCY1* gene has been reported to eliminate the transient accumulation of unbudded cells brought about by heat shock. This finding suggests that cAMP regulation of A kinase activity may be involved in START inhibition after heat shock (42).

We determined the heat shock response of *bcy1* mutant cells completely lacking the Bcy1 regulatory activity because of disruption of the *BCY1* gene. Each Δ*bcy1* cell also contained only a single version of the three redundant cAMP-dependent protein kinase (*TPK*) catalytic subunits (33, 47), with catalytic activity attenuated by a *tpk*^{w} mutation (33) (Table 1). The Δ*bcy1 tpk1*^{w} and Δ*bcy1 tpk2*^{w} mutant cells with attenuated kinase activity still underwent transient START inhibition after heat shock (Fig. 6A, and data not shown), and Δ*bcy1 tpk*^{w} Δ*cln3* cells were similarly inhibited (Fig. 6C). The behavior of the Δ*bcy1 tpk*^{w} cells shows that a heat-induced inhibition of START can take place without cAMP modulation of A kinase activity.

The Δ*bcy1 tpk*^{w} cells were similar to wild-type cells in decreases in *CLN1* and *CLN2* transcript abundance (compare Fig. 6B with Fig. 1B). Thus, the heat shock regulation of *CLN1* and *CLN2* transcript levels is unaffected by this cAMP-independent A kinase activity.

Heat shock gene induction is not affected by cAMP-independent A kinase activity. In addition to showing normal heat shock decreases in *CLN1* and *CLN2* transcript abundances, Δ*bcy1* mutant cells behaved like wild-type cells in heat induction of the *SSA3* heat shock gene (Fig. 6D). Thus, in *bcy1* mutant cells many transcriptional aspects of the heat shock response remain intact.

DISCUSSION

Heat shock induces a variety of changes in yeast cells, including altered gene expression and a transient inhibition of the cell cycle-regulatory step START. The altered gene expression after heat shock includes the inhibition of expression of many genes (16, 40, 50, 51). We show here that two additional genes whose transcript abundance decreases after heat shock are the G_1 cyclin genes *CLN1* and *CLN2*. A third cyclin gene, *CLN3*, was unaffected in transcript abundance by heat shock.

Based on the finding that G_1 cyclin proteins are rate limiting for START (26), it is reasonable to assume that the inhibition of START caused by heat shock may necessitate the inactivation of G_1 cyclins. We have shown that heat shock does in fact decrease expression of the *CLN1* and *CLN2* genes, so that Cln1 and Cln2 protein levels may also be affected by these decreases. The situation for Cln3 is different, because *CLN3* transcript levels are maintained during heat shock. Indirect evidence is consistent with posttranscriptional inhibition of Cln3 activity by heat shock. This suggestion is supported by the recent identification of different roles for the G_1 cyclins in the performance of START. The mode of yeast reproduction results in an asymmetric growth pattern, in which a newly produced daughter cell arises as a bud on the surface of a mother cell. Different G_1 cyclins are implicated in the performance of START in mother and daughter cells; the Cln1 and Cln2 cyclins are necessary for the normal timing of START in daughter cells, while the Cln3 cyclin has a significant role for START in mother cells (26). We have found that both mother and daughter cells accumulate as unbudded cells after heat shock (25), suggesting that the Cln3 activity that is necessary for START in mother cells may be inhibited by heat shock.

The supposition that decreased Cln2 activity is necessary for heat-induced START inhibition is supported by the findings that both the presence of the *CLN2-1* allele and expression of the *CLN2* gene from a heterologous promoter prevented the inhibition of START by heat shock. Likewise, inactivation of Cln3 activity by a posttranscriptional mechanism is consistent with the effects of the *CLN3-1* allele, encoding a stabilized Cln3 protein (48), and with the heat-induced START inhibition, without decreased *CLN3* transcript abundance, in cells lacking functional Cln1 and Cln2 proteins. Although other explanations are possible, including heat shock inhibition of activities downstream of the G_1 cyclins, a simple model is that heat-induced inhibition of START is mediated through effects on cyclins.

The transient inhibition of START is distinct from other aspects of the heat shock response, since even in *CLN2-1* and *CLN3-1* mutant cells that were not inhibited for START, heat shock still resulted in acquired thermotolerance and in transcriptional alterations, such as increased expression of the *SSA3* heat shock gene and decreased abundance of the *CLN1* and *CLN2* transcripts. Thus, the involvement of cyclins during heat shock is limited to effects on START.

For cells to display the effects of a temporary inhibition of START (seen as a transient accumulation of unbudded cells) after heat shock, the heat-shocked cells must be able to complete cell cycles that were in progress at the time of heat shock. For all cases examined here, including situations in which mutant cells did not undergo a transient accumulation of unbudded cells, progress through the post-START cell cycle continued after heat shock, and heat-shocked cells continued to proliferate. Thus, in each case, the absence of an accumulation of unbudded cells was the result of continued START activity.

The heat-induced decreases in *CLN* transcript abundance seen here are analogous to the effects of the pheromone

response pathway. Like heat shock, pheromone signalling also leads to decreased transcript abundance for *CLN1* and *CLN2* but not for *CLN3*. The effects of the pheromone response pathway are achieved in part by the negative regulatory proteins Far1 and Fus3, acting at a transcriptional level (6, 12, 13). We show here that neither the *CLN* transcriptional effects after heat shock nor the heat-induced START inhibition involves Far1 or Fus3 activity. These results indicate that additional regulatory factors account for decreased *CLN1* and *CLN2* transcription after heat shock.

The essential Cdc68 protein, which maintains *CLN1* and *CLN2* transcript levels (38), is unlikely to be a regulatory factor mediating the heat shock effects on these transcripts. Heat shock does not affect *CLN3* transcript abundance (Fig. 1B), whereas decreased Cdc68 function leads to decreased transcript abundance for *CLN3* in addition to *CLN1* and *CLN2*. On the other hand, the Swi4 protein, which is another positive regulator of *CLN1* and *CLN2* gene expression, could be involved in regulation of *CLN* transcript levels during heat shock (32, 34). The absence of Swi4 protein can impose a temperature-sensitive phenotype, so that *swi4* mutant cells accumulate as unbudded cells at 37°C (34). The same temperature also induces the heat shock response. Indeed, heat shock causes a transient but limited decrease in *SWI4* transcript levels, which, through instability of the Swi4 protein (3), could result in decreased Swi4 protein levels. Decreased abundance of this transcription activator may be sufficient to account for the decreased abundance of the *CLN1* and *CLN2* transcripts after heat shock. However, persistent high-level *SWI4* gene expression from the *GAL* promoter did not prevent the heat-induced decrease in *CLN1* and *CLN2* transcript abundance. Therefore, the heat-induced decreases in *SWI4* transcript levels may contribute to decreased *CLN1* and *CLN2* transcript abundance, but heat shock must also inhibit *CLN1* and *CLN2* expression in a manner independent of *SWI4* transcription.

The regulation of cyclin transcription includes a positive feedback loop in which increased cyclin activity stimulates transcription of the *CLN1* and *CLN2* genes, presumably through indirect activation of the Swi4 transcription complex (8, 34). Heat-shocked cells are unresponsive to this positive feedback mechanism, since the presence of hyperstable and/or hyperactive cyclins in *CLN2-1* and *CLN3-1* mutant cells had little effect on heat-induced decreases in *CLN1* or *CLN2* transcript abundance. Thus, heat shock must override any positive feedback on *CLN* gene transcription caused by increased cyclin activity.

Our finding that altered A kinase regulation in Δ*bcy1 tpk*^{w} mutant cells does not affect heat-induced START inhibition is especially informative in light of an earlier observation that cells harboring a *bcy1* point mutation fail to show START inhibition after heat shock (42). We also find that heat-shocked cells harboring *bcy1* point mutations (5) fail to show START inhibition and continue to proliferate and that Δ*bcy1* cells with one intact *TPK* gene behave similarly (25). Therefore, high levels of *TPK*-encoded A kinase activity unregulated by cAMP override the heat shock regulation of START, while attenuated levels of *tpk*^{w}-encoded A kinase activity do not. The ability of unbridled A kinase activity to provoke prompt performance of START after heat shock is not due to altered *CLN* transcriptional regulation; *CLN1* and *CLN2* transcript levels still decrease in heat-shocked Δ*bcy1* mutant cells with an intact *TPK* gene, while *CLN3* transcript levels remain unaffected (39). Instead, high levels of A kinase activity may influence the activity of a Cln protein or a downstream regulator. The involvement of Cln3 is unnecessary, because high levels of unbridled A kinase prevented heat-induced START inhibition even in Δ*cln3* cells (25). The *CDC28* protein kinase functions downstream of the Cln proteins but is unlikely to be a direct target of A kinase, because the Cdc28 protein (28) does not contain an A kinase consensus phosphorylation site (9).

The inhibition of START by heat shock is not seen without some restraint of A kinase by mutation or cAMP-mediated control. The finding that heat shock can inhibit START in the absence of cAMP modulation of A kinase activity points to an effect of heat shock that opposes the effects of A kinase activity, or perhaps to a cAMP-independent modulation of A kinase itself. An example of a regulatory activity that works in opposition to A kinase is that of the Yak1 protein kinase, which is a negative regulator of growth and antagonizes the effects of A kinase (14, 15). The Yak1 kinase is unlikely to be involved in heat-induced START inhibition, however, because mutant cells without Yak1 activity still show heat shock inhibition of START (25).

ACKNOWLEDGMENTS

We thank L. Breeden, J. Brill, J. Cannon, F. Chang, F. Cross, B. Futcher, S. Garrett, R. Storms, M. Wigler, and C. Wittenberg for their generosity in supplying strains and plasmids. We are grateful to members of the Dalhousie and University of New Mexico yeast groups for helpful discussions and for critical reading of the manuscript and to Maggi Kumar for invaluable technical assistance.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (with funds from the Canadian Cancer Society), held jointly by G.C.J. and R.A.S., and grants DCB-9000556 and PYI9058136 to M.W.-W. from the National Science Foundation. G.C.J. is a Senior Research Scientist of the National Cancer Institute of Canada.

REFERENCES

1. **Adams, B. G.** 1972. Induction of galactokinase in *Saccharomyces cerevisiae*: kinetics of induction and glucose effects. J. Bacteriol. **111**:308–315.
2. **Barnes, C. A., G. C. Johnston, and R. A. Singer.** 1990. Thermotolerance is independent of the full spectrum of heat shock proteins and of cell cycle blockage in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **172**:4352–4358.
3. **Breeden, L., and G. E. Mikesell.** 1991. Cell cycle-specific expression of the *SWI4* transcription factor is required for the cell cycle regulation of *HO* transcription. Genes Dev. **5**:1183–1190.

3a. **Brill, J. A.** Personal communication.

4. **Broach, J. R., and R. J. Deschenes.** 1990. The function of *RAS* genes in *Saccharomyces cerevisiae*. Adv. Cancer Res. **54**:79–139.
5. **Cannon, J. F., R. Gitan, and K. Tatchell.** 1990. Yeast cAMP-dependent protein kinase regulatory subunit mutations display a variety of phenotypes. J. Biol. Chem. **265**:11897–11904.
6. **Chang, F., and I. Herskowitz.** 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. Cell **63**:999–1011.
7. **Cross, F. R.** 1990. Cell cycle arrest caused by *CLN* gene deficiency in *Saccharomyces cerevisiae* resembles START-I arrest and is independent of the mating-pheromone signalling pathway. Mol. Cell. Biol. **10**:6482–6490.
8. **Cross, F. R., and A. H. Tinkelenberg.** 1991. A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. Cell **65**:875–883.
9. **Denis, C. L., B. E. Kemp, and M. J. Zoller.** 1991. Substrate specificities for yeast and mammalian cAMP-dependent protein kinases are similar but not identical. J. Biol. Chem. **266**:17932–17935.
10. **Dirick, L., and K. Nasmyth.** 1991. Positive feedback in the activation of G1 cyclins in yeast. Nature (London) **351**:754–757.

11. **Drebot, M. A., C. A. Barnes, R. A. Singer, and G. C. Johnston.** 1990. Genetic assessment of stationary phase for cells of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **172**:3584–3589.
12. **Elion, E. A., J. A. Brill, and G. R. Fink.** 1991. FUS3 represses CLN1 and CLN2 and in concert with KSS1 promotes signal transduction. Proc. Natl. Acad. Sci. USA **88**:9392–9396.
13. **Elion, E. A., P. L. Grisafi, and G. R. Fink.** 1990. *FUS3* encodes a cdc2$^+$/CDC28-related kinase required for the transition from mitosis to conjugation. Cell **60**:649–664.
14. **Garrett, S., and J. Broach.** 1989. Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruption of a new kinase gene, *YAK1*, whose product may act downstream of the cAMP-dependent protein kinase. Genes Dev. **3**:1336–1348.
15. **Garrett, S., M. M. Menold, and J. R. Broach.** 1991. The *Saccharomyces cerevisiae YAK1* gene encodes a protein kinase that is induced by arrest early in the cell cycle. Mol. Cell. Biol. **11**:4045–4052.
16. **Gorenstein, C., and J. R. Warner.** 1976. Coordinate regulation of the synthesis of eukaryotic ribosomal proteins. Proc. Natl. Acad. Sci. USA **73**:1547–1551.
17. **Hadwiger, J. A., C. Wittenberg, H. E. Richardson, M. de Barros Lopes, and S. I. Reed.** 1989. A family of cyclin homologs that control the G1 phase in yeast. Proc. Natl. Acad. Sci. USA **86**:6255–6259.
18. **Hartwell, L. H.** 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. **93**:1662–1670.
19. **Hartwell, L. H.** 1970. Periodic density fluctuation during the yeast cell cycle and the selection of synchronous cultures. J. Bacteriol. **104**:1280–1285.
20. **Hartwell, L. H.** 1974. *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. **38**:164–198.
21. **Henle, K. J., and L. A. Dethlefsen.** 1978. Heat fractionation and thermotolerance: a review. Cancer Res. **38**:570–574.
22. **Johnston, G. C., J. R. Pringle, and L. H. Hartwell.** 1977. Coordination of growth and cell division in the budding yeast *Saccharomyces cerevisiae*. Exp. Cell Res. **105**:79–98.
23. **Johnston, G. C., and R. A. Singer.** 1978. RNA synthesis and control of cell division in the yeast S. cerevisiae. Cell **14**:951–958.
24. **Johnston, G. C., and R. A. Singer.** 1980. Ribosomal precursor RNA metabolism and cell division in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **178**:357–360.
25. **Johnston, G. C., and R. A. Singer.** Unpublished data.
26. **Lew, D. J., N. J. Marini, and S. I. Reed.** 1992. Different G1 cyclins control the timing of cell cycle commitment in mother and daughter cells of the budding yeast S. cerevisiae. Cell **69**:317–327.
27. **Lindquist, S.** 1986. The heat-shock response. Annu. Rev. Biochem. **55**:1151–1191.
28. **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 (London) **307**:183–185.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **McAlister, L., and D. B. Finkelstein.** 1980. Heat shock proteins and thermal resistance in yeast. Biochem. Biophys. Res. Commun. **93**:819–824.
31. **Nash, R., G. Tokiwa, S. Anand, K. Erickson, and A. B. Futcher.** 1988. The *WHI1*$^+$ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. EMBO J. **7**:4335–4346.
32. **Nasmyth, K., and L. Dirick.** 1991. The role of *SWI4* and *SWI6* in the activity of G1 cyclins in yeast. Cell **66**:995–1013.
33. **Nikawa, J., S. Cameron, T. Toda, K. M. Ferguson, and M. Wigler.** 1987. Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*. Genes Dev. **1**:931–937.
34. **Ogas, J., B. J. Andrews, and I. Herskowitz.** 1991. Transcriptional activation of *CLN1*, *CLN2*, and a putative new G1 cyclin (*HCS26*) by SWI4, a positive regulator of G1-specific transcription. Cell **66**:1015–1026.
35. **Penn, M. D., G. Thireos, and H. Greer.** 1984. Temporal analysis of general control of amino acid biosynthesis in *Saccharomyces cerevisiae*: role of positive regulatory genes in initiation and maintenance of mRNA derepression. Mol. Cell. Biol. **4**:520–528.
36. **Reed, S. I.** 1991. G1-specific cyclins: in search of an S-phase-promoting factor. Trends Genet. **7**:95–99.
37. **Richardson, H. E., C. Wittenberg, F. Cross, and S. I. Reed.** 1989. An essential G1 function for cyclin-like proteins in yeast. Cell **59**:1127–1133.
38. **Rowley, A., G. C. Johnston, and R. A. Singer.** 1991. *CDC68*, a yeast gene that affects regulation of cell proliferation and transcription, encodes a protein with a highly acidic carboxyl terminus. Mol. Cell. Biol. **11**:5718–5726.
39. **Rowley, A., G. C. Johnston, and R. A. Singer.** Unpublished data.
40. **Schlesinger, M. J., M. Ashburner, and A. Tissieres.** 1982. Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18**:3091–3092.
42. **Shin, D.-Y., K. Matsumoto, H. Iida, I. Uno, and T. Ishikawa.** 1987. Heat shock response of *Saccharomyces cerevisiae* mutants altered in cyclic AMP-dependent protein phosphorylation. Mol. Cell. Biol. **7**:244–250.
43. **Singer, R. A., and G. C. Johnston.** 1981. Nature of the G1 phase of the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78**:3030–3033.
44. **Sudbery, P. E., A. R. Goodey, and B. L. A. Carter.** 1980. Genes which control cell proliferation in the yeast *Saccharomyces cerevisiae*. Nature (London) **288**:401–404.
45. **Thomas, P. S.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
46. **Toda, T., S. Cameron, P. Sass, M. Zoller, J. D. Scott, B. McMullen, M. Hurwitz, E. G. Krebs, and M. Wigler.** 1987. Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:1371–1377.
47. **Toda, T., S. Cameron, P. Sass, M. Zoller, and M. Wigler.** 1987. Three different genes in Saccharomyces cerevisiae encode the catalytic subunits of the cyclic AMP-dependent protein kinase. Cell **50**:277–288.
48. **Tyers, M., G. Tokiwa, R. Nash, and B. Futcher.** 1992. The Cln3-Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. **11**:1773–1784.
49. **Veinot-Drebot, L. M., G. C. Johnston, and R. A. Singer.** 1991. A cyclin protein modulates mitosis in the budding yeast *Saccharomyces cerevisiae*. Curr. Genet. **19**:15–19.
50. **Veinot-Drebot, L. M., R. A. Singer, and G. C. Johnston.** 1989. Heat shock causes transient inhibition of yeast rRNA gene transcription. J. Biol. Chem. **264**:19473–19474.
51. **Werner-Washburne, M., J. Becker, J. Kosic-Smithers, and E. A. Craig.** 1989. Yeast Hsp70 RNA levels vary in response to the physiological status of the cell. J. Bacteriol. **171**:2680–2688.
52. **Werner-Washburne, M., D. E. Stone, and E. A. Craig.** 1987. Complex interactions among members of an essential subfamily of *hsp70* genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:2568–2577.
53. **Wittenberg, C., K. Sugimoto, and S. I. Reed.** 1990. G1-specific cyclins of S. cerevisiae: cell cycle periodicity, regulation by mating pheromone, and association with the p34^{CDC28} protein kinase. Cell **62**:225–237.