MOLECULAR AND CELLULAR BIOLOGY, Aug. 1991, p. 4045–4052
0270-7306/91/084045-08$02.00/0

Vol. 11, No. 8

The *Saccharomyces cerevisiae YAK1* Gene Encodes a Protein Kinase That Is Induced by Arrest Early in the Cell Cycle

STEPHEN GARRETT,[1]* MARISA M. MENOLD,[1] AND JAMES R. BROACH[2]

Section of Cell Growth, Regulation and Oncogenesis and Department of Biochemistry, Box 3686, Duke University Medical Center, Durham, North Carolina 27710,[1] and Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544[2]

Received 26 February 1991/Accepted 16 May 1991

Null mutations in the gene *YAK1*, which encodes a protein with sequence homology to known protein kinases, suppress the cell cycle arrest phenotype of mutants lacking the cyclic AMP-dependent protein kinase (A kinase). That is, loss of the Yak1 protein specifically compensates for loss of the A kinase. Here, we show that the protein encoded by *YAK1* has protein kinase activity. Yak1 kinase activity is low during exponential growth but is induced at least 50-fold by arrest of cells prior to the completion of S phase. Induction is not observed by arrest at stages later in the cell cycle. Depending on the arrest regimen, induction can occur either by an increase in Yak1 protein levels or by an increase in Yak1 specific activity. Finally, an increase in Yak1 protein levels causes growth arrest of cells with attenuated A kinase activity. These results suggest that Yak1 acts in a pathway parallel to that of the A kinase to negatively regulate cell proliferation.

The cyclic AMP-dependent protein kinase (A kinase) pathway is required for cell cycle progression in the yeast *Saccharomyces cerevisiae*. In the absence of A kinase activity, cells cease to grow and arrest in G_1 in a manner similar to that observed following starvation of cells for essential nutrients (21, 24, 30). In contrast, mutations yielding elevated or unrestricted A kinase activity prevent cells from arresting in G_1 following nutrient starvation or heat shock (8, 38). In addition, such mutations cause loss of carbohydrate reserves, sporulation deficiency, and sensitivity to heat shock or to prolonged starvation for nutrients. These phenotypes have been taken as evidence that A kinase participates in the cell's decision to enter a quiescent G_0 state. High activity precludes access to G_0, and low activity promotes exit from the mitotic cycle and entry into G_0 (for a review, see reference 13).

The activity of A kinase is regulated by an elaborate process mediated by the yeast homologs of mammalian *ras* genes (2). The yeast *RAS1* and *RAS2* products (referred to hereafter as Ras), like mammalian *ras* proteins and a large family of structurally related proteins, are GTP-binding proteins that possess an intrinsic but weak GTPase activity. When bound to GTP, Ras stimulates adenylate cyclase, the product of the *CYR1* locus (22, 35). The primary if not exclusive role of cyclic AMP in the cell is regulation of the A kinase. The A kinase is composed of two regulatory subunits, encoded by *BCY1*, and two catalytic subunits, encoded redundantly by three genes, *TPK1*, *TPK2*, and *TPK3* (8, 36). Binding of cyclic AMP to the Bcy1 protein alleviates Bcy1 inhibition of the catalytic subunit and thereby reveals kinase activity.

The precise mechanism by which the A kinase influences the cell's decision between quiescence and growth is not known. Targets of the A kinase include enzymes involved in metabolism of storage carbohydrates, enzymes situated a strategic points in the glycolytic/gluconeogenic pathway, enzymes required for phospholipid metabolism, transcription factors associated with expression of specific genes, and proteins involved in production of cyclic AMP itself (10, 29, 34, 39). However, whether A kinase regulation of these known targets can account for the decision to continue growing or to exit the mitotic cycle remains unclear (7).

To examine how A kinase influences cell proliferation, we previously conducted a genetic screen to identify novel components that might act downstream of the A kinase. By isolating revertants of strains diminished in A kinase activity, we identified a gene, *YAK1*, that exhibited significant sequence homology to known protein kinases (16, 18). We observed that null mutations of *YAK1* allowed strains completely deficient in A kinase to grow; *tpk1 tpk2 tpk3 YAK1*$^+$ strains are inviable, but *tpk1 tpk2 tpk3 yak1* strains grow. That is, viability is restored to strains lacking the A kinase by elimination of yet another kinase, Yak1.

The properties of *yak1* strains led us to propose that Yak1 acts specifically to inhibit cell growth (16). Yak1 could directly mediate the A kinase growth control by serving as a negative regulator of cell growth that could be inactivated by phosphorylation by the A kinase. Alternatively, Yak1 could participate in a pathway parallel to that of the A kinase, with overlapping targets but antagonistic effects. Here we provide confirmation of this latter hypothesis. We show that Yak1 functions as a negative regulator of cell growth. In addition, our results suggest that Yak1 protein and the A kinase are components of parallel, interdependent pathways.

MATERIALS AND METHODS

Media and growth conditions. Most media used (yeast rich and minimal media and bacterial media) were prepared as described previously (12). Nitrogen-poor medium (16) contained 0.17% yeast nitrogen base without ammonium sulfate or amino acids (Difco) and 2% glucose as a carbon source. Cells were labelled with $^{32}P_i$ in yeast rich medium that had been pretreated with ammonium sulfate to precipitate P_i (31). Nocodazole was added to a final concentration of 15 μg/ml as described previously (25). The mating pheromone α-factor (Sigma) was stored as a 100 μM stock solution in methanol and added to exponentially growing cells (*MAT***a** *bar1*::*LEU2*) to a final concentration of 1 μM. Cultures, with

* Corresponding author.

TABLE 1. Yeast strains

Strain	Genotype	Source or reference
SGP4	*MATα leu2-3,112 trp1 ura3-52 his3 ade8 ras1::HIS3 RAS2-URA3*	16
SGP34	*MATα leu2-3,112 trp1 ura3-52 his3 ade8 ras1::HIS3 ras2-34*(Ts)-*URA3*	16
SGP400	*MATa leu2-3,112 trp1 ura3-52 his3 ade8 yak1-1::LEU2*	16
SGY41	SGP400/YEp24	This study
SGY42	SGP400/pGS127	This study
SGY43	SGP400/pGS161	This study
SGY95	*MATa cyr1-1 ura3-52*	This study
SGY15	*MATa cdc28-1 leu2 lys2 his4Δ34*	This study
SGY27	*MATa cdc33-1 ura3 leu2*	This study
SGY4	*MATa cdc63-1*	This study
SGY290	*MATa ura3-52 his leu2-3,112 trp1 ade2-101*(Oc) *lys2-810*(Am)::*lys2-GAL10-lacZ bar1::LEU2*	This study
H8C141	*MATa his7 ura1 cdc8-1*	Lee Hartwell
H15C2A2	*MATa his7 ura1 cdc15-2*	Lee Hartwell
H20C1A1	*MATa his7 ura1 cdc20-1*	Lee Hartwell

or without added α-factor, were incubated at 30°C for 4 h and then used to prepare extracts.

Strains and plasmids. Yeast strains are listed in Table 1. Bacterial strains were MC1066 [Δ(*lac*)*X74 galU galK strA hsdR trpC9830 leuB6 pyrF*::Tn*5*] and JM101 [Δ(*lac-pro*) *supE thi*/F' *traD36 proAB lacI*^{q}*Z ΔM15*] and have been described elsewhere (9, 27). Phage mpG6 was constructed by inserting the 2.0-kbp *Bam*HI-*Hin*dIII fragment of *YAK1* (16) into the corresponding sites of mp11 (27). The high-copy-number yeast vector YEp24 has been described previously (4). The *lacZ* vector (pTRB0) used to construct the LacZ-Yak1 protein fusions was made by T. Burglin and has been described elsewhere (41).

DNA manipulations. Plasmid DNA was prepared from *Escherichia coli* by the alkali lysis method (23). All enzymes were used according to the specifications of the supplier (New England BioLabs or Bethesda Research Laboratories [BRL]), and cloning techniques were as described previously (23). The lysine (K) at position 398 was changed to a tyrosine (Y) residue by site-directed mutagenesis according to the procedure of Zoller and Smith (42). The oligonucleotide 5'-ATTGGCTGTATACGTGGTTAAAT-3' was hybridized to its complementary single-stranded sequence (mismatches are underlined) in phage mpG6. Plaques were screened by hybridization with the labelled oligonucleotide, followed by digestion of double-strand (replicative-form) DNA with *Acc*I. An *Acc*I site is formed by the substitution of both underlined nucleotides for A residues normally found in those positions. The altered sequence was then cloned into the appropriate vectors, using the naturally occurring *Bam*HI and *Sal*I sites of *YAK1*.

Plasmid pGS127 consists of a 5.6-kbp *Nhe*I-*Bgl*II fragment (*YAK1*$^+$) from pGS100 (16) cloned into the corresponding sites of the high-copy-number *URA3* vector YEp24. The variant pGS161 is the same plasmid containing the K398-to-Y398 alteration. Plasmid pGS158 was constructed by replacing the 700-bp *Bam*HI-*Eco*RV fragment of the high-copy-number vector YEp*ADE8* (provided by T. Toda and S. Cameron) with the 5.6-kbp *Bgl*II-*Nhe*I *YAK1* fragment (the *Nhe*I site was first made blunt by a fill-in reaction).

The *lacZ-YAK1* fusion plasmid pGS141 was constructed by digesting plasmid pTRB0 with *Bam*HI, filling in the 5' extensions with Klenow enzyme and the four deoxynucleoside triphosphates, digesting the plasmid with *Hin*dIII, and then inserting the 1.1-kbp *Eco*RV-*Hin*dIII fragment of *YAK1* into the appropriate sites. The insertion of this sequence into pTRB0 created an in-frame fusion between most of β-galactosidase and the complete C-terminal 219 amino acids of Yak1. This region of the Yak1 protein does not contain any of the conserved kinase domains.

Preparation of Yak1 antisera. Induction, purification, and preparation of the LacZ-Yak1 fusion protein for injection into New Zealand White rabbits were done essentially as described previously (41). Polyclonal antibodies were prepared as described previously (19) and used without further purification.

Labelling of cells. Yeast proteins were labelled in vivo with [^{35}S]methionine or $^{32}P_i$ according to published procedures (12, 16, 37), and immunoprecipitations were conducted as described previously (12) except for the addition of 0.5% sodium dodecyl sulfate (SDS) in extracts where noted.

Immune complex reactions. Immune complex reactions were done with a total of 500 μg of protein per immunoprecipitation and, except where noted, with 1 μl of undiluted polyclonal antibody. Cells (50 ml) were harvested during exponential phase or after 3 to 5 h under conditions for arrest, washed in sorbitol wash buffer (12), and then disrupted by vortexing in 1 ml of sorbitol lysis buffer (sorbitol wash buffer with 1 mM phenylmethylsulfonyl fluoride, 10 U of aprotinin per ml, and 50 mM NaF) and glass beads. Extracts were clarified by centrifugation twice in a microfuge at 13,000 rpm, 15 min each time. Clarified extracts were then used directly or stored at −80°C. After incubation of the antibody with the extracts at 4°C for 1 h, 20 μl of protein A agarose (BRL) was added to the reaction mix and incubation was continued for an additional hour at 4°C. The immune complex was then washed three times with Hermann's buffer (1% Nonidet P-40, 0.5% deoxycholate, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.2) and twice with 100 mM NaCl–10 mM Tris, pH 7.4, and then resuspended in 25 μl of reaction buffer, which consisted of 10 mM Tris (pH 7.4), 50 mM NaF, 200 μg of casein per ml, 10 mM cold ATP, 1 mM $MgCl_2$, and 400 μCi of [γ-^{32}P]ATP (ICN) per ml. After 10 min at room temperature, the reactions were stopped by adding gel loading buffer and boiling for 5 min. To visualize Yak1 and casein phosphorylation, samples were separated on 7.5 or 10% polyacrylamide gels.

A kinase phosphorylation of Yak1. In vitro phosphorylation of Yak1 by bovine A kinase was carried out after immunoprecipitation of Yak1 or Yak1^{K398Y} from extracts. A kinase catalytic subunit (1 U; Sigma) was added to the immune complex along with either unlabelled ATP (10 mM) or [γ-^{32}P]ATP (400 μCi/ml) and, where indicated, A kinase inhibitor (10 μg/ml; Sigma) and casein (200 μg/ml). Reaction

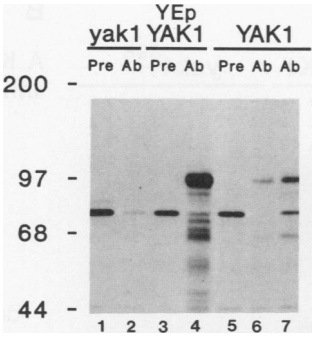

FIG. 1. Identification of Yak1 protein by immunoprecipitation. Extracts of [^{35}S]methionine-labelled cells were prepared as described in Materials and Methods and mixed with preimmune (Pre) or anti-Yak1 (Ab) serum. Immunoprecipitates were collected and washed as described in Materials and Methods and then separated on a 7.5% polyacrylamide gel. Immunoprecipitations were performed in 500-μl aliquots, using 1 μl of polyclonal serum (with the exception of lane 7, in which 3 μl of serum was used). Strains were SGY41 (*yak1*), SGY42(YEpYAK1), and SGP4 (*YAK1*). The standards used in all figures to determine molecular sizes (positions indicated in kilodaltons here and in Fig. 2, 3, and 6) were prestained high-molecular-weight markers from BRL (in Fig. 4 and 5, the positions of the standards are not marked).

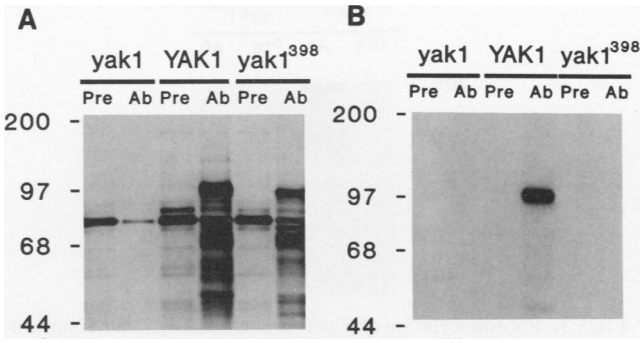

FIG. 2. Evidence that Yak1 catalyzes autophosphorylation in vitro. (A) Extracts of [^{35}S]methionine-labelled cells were prepared and treated as described in the legend to Fig. 1. Immunoprecipitates were fractionated on a 7.5% polyacrylamide gel. (B) Protein extracts from unlabelled cells were immunoprecipitated as in panel A with preimmune (Pre) or anti-Yak1 (Ab) antibody. Immunoprecipitates were incubated with [γ-^{32}P]ATP as described in Materials and Methods, washed, and then fractionated by PAGE. Strains were all constructed by transforming SGP400 (*yak1*) with YEp24 containing the *YAK1* allele indicated in parentheses: SGY41 (*yak1*), SGY42 (*YAK1*), and SGY43 (*yak1*^{398}).

mixtures were incubated for 20 min at 30°C, and the reactions were stopped by boiling in loading buffer. When the effect of A kinase phosphorylation on Yak1 activity was monitored, reactions were terminated by several washes with reaction buffer, and then the mixtures were incubated with casein and [γ-^{32}P]ATP as described above.

Immunological techniques. For Western immunoblot analysis, extracts (500 μg) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to reinforced nitrocellulose by electrophoresis (19). The filter was incubated with anti-Yak1 antibody at a 1/300 dilution, followed by either goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma) or goat anti-rabbit antibody conjugated to biotin. In the latter case, the filter was then incubated with alkaline phosphatase conjugated to avidin (BRL).

RESULTS

Yak1 is a protein kinase. The nucleotide sequence of *YAK1* predicts that it encodes a 97-kDa protein with extensive homology to known protein kinases. Over a 280-amino-acid region, the *YAK1* sequence is 32% identical to the catalytic domains of the *S. cerevisiae* Cdc28 and *Schizosaccharomyces pombe* Cdc2 protein kinases (16). In particular, those amino acid sequences strictly conserved among known protein kinases (18) are present within the predicted *YAK1* sequence. This sequence similarity led us to examine whether Yak1 is a protein kinase.

We raised polyclonal antibodies against a β-galactosidase–Yak1 fusion protein purified from *E. coli*. As shown in Fig. 1, this serum recognizes a 97-kDa protein from extracts of a *YAK1*$^+$ strain that is absent from extracts of an isogenic *yak1* strain. The amount of this protein is present at increased levels in extracts of a strain harboring a multicopy plasmid carrying *YAK1* (lane 4). These results demonstrate that the serum contains antibodies that recognize Yak1 and that Yak1 is the 97 kDa protein specifically precipitated by the serum.

To assess whether Yak1 has protein kinase activity, we determined whether Yak1 could phosphorylate itself in vitro. We immunoprecipitated Yak1 from an extract of an unlabelled *YAK1* strain and incubated it with [γ-^{32}P]ATP. This procedure resulted in phosphorylation of a 97-kDa protein (Fig. 2B). To eliminate the possibility that in vitro Yak1 phosphorylation was catalyzed by a protein kinase that coprecipitates with Yak1, we examined the in vitro autophosphorylation activity of an ATP-binding-deficient *yak1* mutant. We introduced a mutation in *YAK1* to convert lysine 398 to tyrosine. This lysine residue corresponds to the conserved lysine shown to be required for ATP binding and, accordingly, kinase activity of a variety of known protein kinases (18). The resulting mutant *YAK1* gene, *yak1*^{K398Y}, fails to complement *yak1* mutations (data not shown), even though the 97-kDa, Yak1-specific protein is present in strains carrying the mutant allele (Fig. 2A, yak1^{398}). Incubation of [γ-^{32}P]ATP with an immunoprecipitate obtained with our anti-Yak1 serum and an extract of a *yak1*^{K398Y} strain failed to yield any detectable phosphorylation of a 97-kDa protein (Fig. 2B). This result confirms that in vitro phosphorylation of the Yak1 protein results from an autocatalytic reaction and, accordingly, that Yak1 is a protein kinase.

We also examined whether Yak1 protein could phosphorylate various exogenous substrates. Immunoprecipitates of Yak1 were unable to phosphorylate histone H1 or enolase. However, casein could be efficiently phosphorylated by immunoprecipitates of Yak1 protein (see, for example, Fig. 5). Phosphorylation activity required either Mg^{2+} or Mn^{2+} as a divalent cation; neither Ca^{2+} nor Zn^{2+} could substitute.

Yak1 is phosphorylated in vivo by other protein kinases. One model to account for the behavior of *yak1* mutants postulates that Yak1 serves to inhibit cell growth but that Yak1 activity can be attenuated by phosphorylation by the A kinase. This model was prompted in part by the presence of four A kinase consensus phosphorylation sites within the nonconserved amino half of Yak1. To test this model, we examined whether Yak1 is phosphorylated in vivo and, if so, what effects this phosphorylation has on Yak1 protein kinase activity.

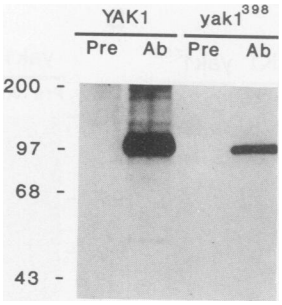

FIG. 3. Phosphorylation of Yak1 by itself and by other kinases in vivo. Extracts from strains labelled with $^{32}P_i$ were immunoprecipitated with preimmune (Pre) or anti-Yak1 (Ab) antibody. Immunoprecipitates were fractionated on a 7.5% polyacrylamide gel. Strains were SGY42 (*YAK1*) and SGY43 (*yak1*^{398}) (see Fig. 2).

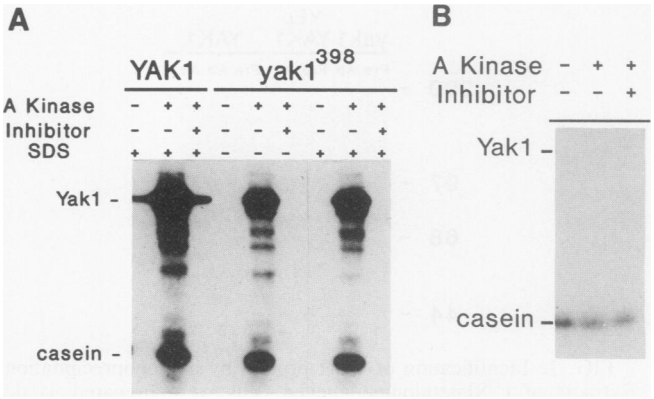

FIG. 4. Evidence that Yak1 protein is a substrate for the A kinase in vitro. (A) Extracts from unlabelled cells were prepared as described in Materials and Methods. Immunoprecipitation with anti-Yak1 antiserum was performed on native extract or on extract denatured by addition of 0.5% SDS, as indicated (the presence of 0.5% SDS does not inhibit immunoprecipitation of Yak1 protein by the anti-Yak1 antiserum). Immunoprecipitates were incubated with [^{32}P]ATP and casein and either with no further additions, with A kinase, or with A kinase plus A kinase inhibitor, as indicated. (B) Yak1 protein was immunoprecipitated with anti-Yak1 antibody from an extract of strain SGP4 (*YAK1*), prepared from asynchronous cells following starvation of the strain for nitrogen as described in Materials and Methods. Identical immunoprecipitated samples were incubated with unlabelled ATP and either with no further additions, with A kinase, or with A kinase plus A kinase inhibitor, as indicated. The samples were washed extensively, incubated with casein, A kinase inhibitor, and [^{32}P]ATP, and then fractionated on a 10% polyacrylamide gel.

As the first step in this analysis, we determined that Yak1 is phosphorylated in vivo both by itself and by other cellular kinases. Exponentially growing *YAK1* and *yak1*^{K398Y} strains were labelled with $^{32}P_i$. Yak1 was immunoprecipitated from extracts of labelled cells and fractionated by SDS-PAGE. As shown in Fig. 3, Yak1 protein from a *YAK1* strain is extensively phosphorylated in vivo, while Yak1^{K398Y} from the *yak1*^{K398Y} strain is also phosphorylated in vivo, but to a lesser extent than seen for Yak1 (Fig. 2A shows the relative amounts of the two proteins). As is evident from the [^{35}S] methionine-labelled cultures, the steady-state level of Yak1^{K398Y} is significantly lower than that of wild-type Yak1 protein.

We interpret these results to indicate that Yak1 undergoes extensive autophosphorylation in vivo. Since the mutant protein, which is unable to catalyze autophosphorylation, is phosphorylated in vivo, Yak1 must also serve as a substrate for other protein kinases in the cell. Finally, to a first approximation, the extent of in vivo phosphorylation of Yak1^{K398Y} appears to be equivalent. The diminished amount of phosphorylated Yak1 protein in *yak1*^{K398Y} versus *YAK1* strains reflects primarily a diminished level of Yak1 protein as well as the absence of autophosphorylation in the mutant strain.

To determine the role of the A kinase in Yak1 phosphorylation, we examined the ability of the A kinase to phosphorylate Yak1^{K398Y} both in vitro and in vivo. Incubation of immunoprecipitated, SDS-treated Yak1^{+} protein with bovine protein kinase A (Sigma) and [^{32}P]ATP led to extensive phosphorylation of the Yak1 protein (Fig. 4A). Similar results were obtained with native or denatured Yak1^{K398Y} protein. This finding indicates that A kinase phosphorylates Yak1 in vitro. To determine whether it actually does so in the cell, we immunoprecipitated Yak1 protein from $^{32}P_i$-labelled *yak1*^{K398Y} *TPK*$^{+}$ and *yak1*^{K398Y} *tpk1 tpk2 tpk3* strains. We observed phosphorylation of Yak1 protein from both strains, although the level of Yak1 phosphorylation in the A kinase-deficient strain varied considerably with each experiment (data not shown). While this variation may be due to the considerably slower growth of the *tpk* strain, it has made it impossible to determine unequivocally whether Yak1 protein is a substrate of the A kinase in vivo. Nevertheless, these results suggest that if Yak1 is phosphorylated by the A kinase, it serves as a substrate for at least one other kinase as well.

To address the significance of A kinase phosphorylation of Yak1, we examined the kinase activity of Yak1 protein before and after treatment with bovine A kinase in vitro. As seen from the results presented in Fig. 4B, the ability of Yak1 to phosphorylate casein is undiminished by preincubation with bovine A kinase. Results similar to those shown in Fig. 4B have been observed with use of Yak1 isolated from *ras*(Ts) and nutrient-starved cells (data not shown). Thus, in this context, phosphorylation by A kinase in vitro has little effect on the kinase activity of Yak1.

Yak1 kinase activity is induced by arrest early in the cell cycle. If Yak1 acts as a negative regulator of growth, then we might anticipate that Yak1 activity should increase coincident with cessation of cell growth. Accordingly, we used the Yak1 immune complex assay described above, monitoring casein phosphorylation as a measure of Yak1 kinase activity, to examine Yak1 activity under various conditions causing growth arrest. These experiments were conducted with strains in which *YAK1* was present at single copy at its normal chromosomal location. The results of this analysis are presented in Fig. 5.

We first examined the effects on Yak1 activity of arresting growth in G_1. As shown in Fig. 5A, *YAK1* cells arrested in G_1 by nitrogen starvation exhibit a significant increase in Yak1-catalyzed casein phosphorylation. Enhanced casein kinase activity is Yak1 specific, since an isogenic *yak1*::*LEU2* strain fails to yield immune complexes with appreciable casein phosphorylation activity under the same conditions. Preimmune serum also fails to precipitate an activity from nitrogen-starved *YAK1* cells capable of phosphorylating casein (data not shown).

Similar results were obtained by incubating G_1-specific, temperature-sensitive *cdc* mutants at the nonpermissive temperature (Fig. 5B). For example, a *ras1* strain carrying a

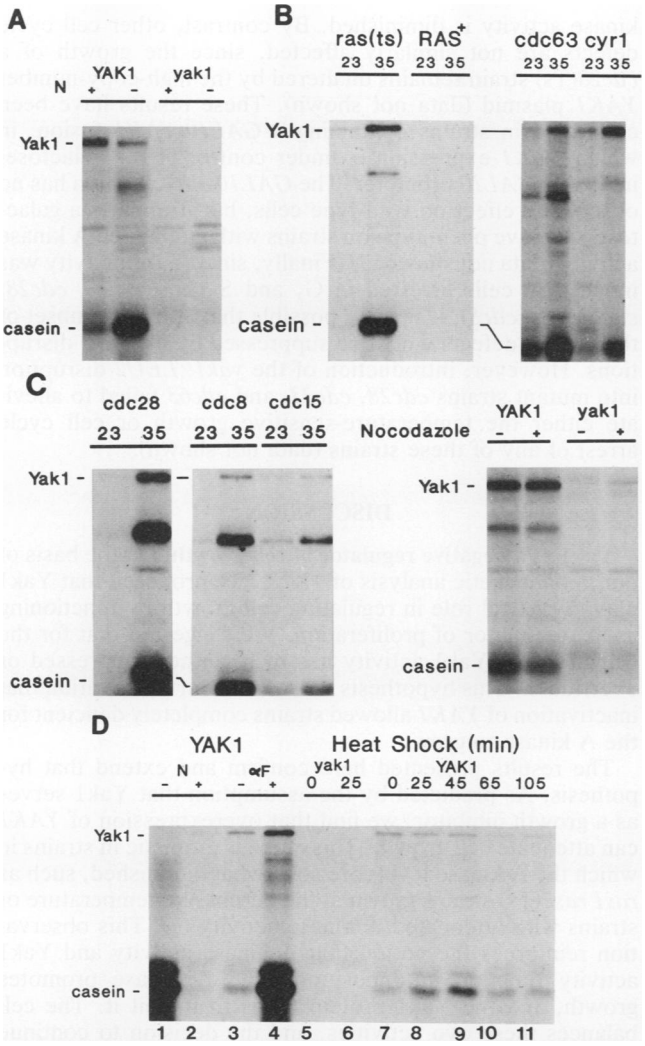

FIG. 5. Induction of Yak1 protein kinase activity by arrest early in the cell cycle. Extracts of strains grown as indicated below were incubated with anti-Yak1 antiserum. Immunoprecipitates were collected, washed, and then incubated with casein and [^{32}P]ATP as indicated in Materials and Methods. Reactions were terminated by addition of sample buffer, and the samples were then fractionated on 10% polyacrylamide gels. The positions of migration of casein and Yak1 protein are indicated. (A) Arrest by nitrogen starvation. Strains were grown in synthetic complete medium to 10^6 cells per ml and then shifted to nitrogen-poor medium supplemented (+) or not supplemented (−) with ammonium sulfate (nitrogen) as described in Materials and Methods. Cells were harvested 6 h after the shift. Strains were SGP4 (*YAK1*) and SGP400 (*yak1*). (B) Arrest of G_0-specific cell cycle mutants. Cultures of the indicated strains were grown at 23°C to 10^6 cells per ml and then divided into two cultures, one of which was continued at 23°C and the other shifted to 35°C. Cells were harvested 3 h after the shift. Strains were SGP34 [*ras*(Ts)], SGP4 (*RAS*$^+$), SGY4 (*cdc63*), and SGY95 (*cyr1*). (C) Arrest at stages other than G_0. Temperature-sensitive strains were treated as in panel B. *YAK1* and *yak1* strains were grown at 30°C in YEPD medium to 10^6 cells per ml, and then incubation was continued for 3 h in the presence (+) or absence (−) of nocodazole (25). Strains were SGY16 (*cdc28*), H8C141 (*cdc8*), H15C2A2 (*cdc15*), SGP4 (*YAK1*), and SGP400 (*yak1*). (D) Arrest by nitrogen starvation, pheromone addition, and heat shock. Cultures were grown at 30°C and starved for N (as in panel A) or arrested for 4 h by the addition of α-factor as described in Materials and Methods. Strains were SGP4 (*YAK1*, N) and SGY290 (*YAK1*, αF). For heat shock, cultures were incubated at 22°C and then shifted to 38°C for the indicated times (minutes). Strains were SGP400 (*yak1*) and SGP4 (*YAK1*).

temperature-sensitive *ras2* allele (16) shows a substantial increase in Yak1 activity upon shift to the nonpermissive temperature. This increase does not occur in an isogenic *RAS2* strain, arguing that a temperature shift per se does not stimulate Yak1 activity. Similarly, a strain carrying a temperature-sensitive *cyr1* allele also exhibits an increase in Yak1 activity at the nonpermissive temperature. Induction of Yak1 activity is not strictly dependent on inactivation of the Ras/A kinase pathway, though. Two other mutant strains, carrying *cdc63* (17) and *cdc33* (6) temperature-sensitive alleles, respectively, exhibit G_1 arrest and also show a significant increase in Yak1 activity upon shift to the nonpermissive temperature (Fig. 5B and data not shown). Finally, temperature shift and extended growth at 35°C do not induce Yak1 protein activity (Fig. 5B).

Induction of Yak1 activity can also occur by arresting cells between G_1 and S phase but not by arresting cells after S phase. As shown in Fig. 5C, *cdc28* (late G_1 arrest; 26) and *cdc8* (early S-phase arrest; 30) strains incubated at the nonpermissive temperature have enhanced Yak1 casein kinase activity. However, mitosis-specific mutants *cdc15* and *cdc20* (data not shown) shifted to the elevated temperature do not have enhanced activity (30). Similarly, arresting cells at mitosis with the microtubule-depolymerizing drug nocodazole (20) fails to elicit induction of Yak1 activity (note that the autoradiographic exposure for this experiment is considerably longer than that for the other experiments shown in Fig. 5).

Temperature shift and nitrogen starvation have, to varying degrees, been implicated in the stress response. Thus, one interpretation of the results shown in Fig. 5A to C was that the stress response, and not cell cycle arrest per se, was responsible for the increase in Yak1 activity. The results presented in Fig. 5D argue against this possibility. Addition of the mating pheromone α-factor to *MAT***a** cells elicits a phenotype similar to that exhibited by a *cdc28* strain incubated at the restrictive temperature. It is clear from lanes 3 and 4 of Fig. 5D that α-factor results in an increase in Yak1 activity that is comparable in magnitude to the induction exhibited by *cdc28* (Fig. 5C) and N-starved (Fig. 5A and D) cells. By contrast, conditions that elicit the heat shock response (shift from 22 to 38°C for the times indicated; 33) result in only a moderate and transient increase in Yak1 kinase activity (Fig. 5D, lanes 7 to 11). This increase is clearly insufficient to account for the activation observed on nutrient deprivation or G_1 arrest.

Yak1 activity is induced through two different mechanisms. Several different molecular mechanisms could account for the increase in Yak1 activity seen in cells arrested early in the cell cycle. These can be divided into two broad categories: (i) those that increase the activity of Yak1 through phosphorylation-induced activation, for example, and (ii) those that increase the amount of Yak1, such as activation of *YAK1* transcription, stabilization or enhanced translation of *YAK1* mRNA, or reduced Yak1 turnover. To distinguish between these two categories, we examined the level of Yak1 protein under the conditions used to examine Yak1 activity.

As shown in Fig. 6, for every arrest regimen tested except nitrogen starvation, much of the increase in Yak1 casein kinase activity can be accounted for by an increase in the in vivo level of Yak1. Since we cannot detect, by Western blot, uninduced levels of Yak1 in single-copy *YAK1* strains, we cannot determine the absolute degree of Yak1 induction. However, the value is certainly commensurate with the fold

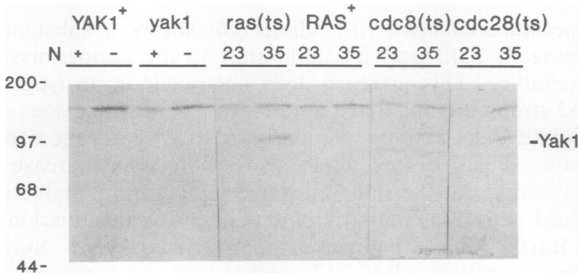

FIG. 6. Analysis of Yak1 protein levels under conditions of induction of Yak1 activity. Extracts were prepared from strains grown as described in the legend to Fig. 5. Samples of the extracts were fractionated on a 7.5% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-Yak1 serum as described in Materials and Methods. The position of migration of Yak1 protein is indicated. The upper band in all lanes is seen by preimmune serum and is not related to Yak1. Strains were as indicated in the legend to Fig. 5.

increase in casein kinase activity seen under the same conditions.

In contrast to these results, induction of Yak1-specific casein kinase activity upon starvation for a nitrogen source is not accompanied by an increase in Yak1 levels (Fig. 6). This finding suggests that induction occurs by activation of the protein, most likely through some posttranslational modification. Consistent with this observation, the extent of Yak1 in vitro autophosphorylation following nitrogen starvation is less than that seen with protein obtained prior to starvation (Fig. 5A). This suggests that Yak1 isolated post-starvation contains fewer unphosphorylated sites than does protein isolated prior to starvation.

Overproduction of Yak1 protein can inhibit cell growth. If Yak1 participates in suppressing cell proliferation, then we would predict that its overexpression should cause growth arrest. Under certain circumstances, this prediction is fulfilled. For instance, the growth of *ras1* strains carrying a *ras2* temperature-sensitive allele are inhibited at the permissive temperature by the presence of a high-copy-number *YAK1* plasmid. In the absence of *YAK1*, such strains are viable under these conditions. Isogenic strains with a wild-type *RAS2*$^+$ gene are not inhibited by the presence of either plasmid. These results (Fig. 7) suggest that inhibition of growth by Yak1 is most pronounced in strains in which A

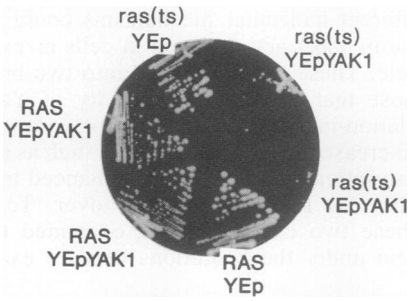

FIG. 7. Evidence that *YAK1* overexpression antagonizes growth of strains with diminished A kinase activity. Strains were transformed either with a high-copy-number vector (YEp; YEp*ADE8*) or with a derivative containing the *YAK1* gene (YEpYAK1; pGS158). Individual colonies were then streaked for single colonies onto selective minimal medium agar at the permissive temperature (23°C). Strains were SGP4 (*RAS*) and SGP34 [*ras*(Ts)].

kinase activity is diminished. By contrast, other cell cycle defects are not similarly affected, since the growth of a *cdc28*(Ts) strain remains unaltered by the high-copy-number *YAK1* plasmid (data not shown). These results have been confirmed in strains containing a *GAL10-YAK1* fusion, in which *YAK1* expression is under control of the galactose-inducible *GAL10* promoter. The *GAL10-YAK1* fusion has no observable effect on wild-type cells, but it imparts a galactose-sensitive phenotype on strains with attenuated A kinase activity (data not shown; 7). Finally, since Yak1 activity was induced in cells arrested in G_1 and S phase (e.g., *cdc28*, *cdc63*, and *cdc8*), it seemed possible that at least a subset of the mutant defects would be suppressed by the *yak1* disruptions. However, introduction of the *yak1*::*LEU2* disruption into mutant strains *cdc28*, *cdc33*, and *cdc63* failed to alleviate either the temperature-sensitive growth or cell cycle arrest of any of these strains (data not shown).

DISCUSSION

Yak1 as a negative regulator of cell growth. On the basis of our initial genetic analysis of *YAK1*, we proposed that Yak1 plays a critical role in regulating cell growth by functioning as an attenuator of proliferation. We suggested that for the cell to grow, Yak1 activity has to be either suppressed or overridden. This hypothesis arose from our observation that inactivation of *YAK1* allowed strains completely deficient for the A kinase to grow.

The results presented here confirm and extend that hypothesis. As predicted by the assumption that Yak1 serves as a growth inhibitor, we find that overexpression of *YAK1* can attenuate cell growth. This effect is dramatic in strains in which the A kinase levels are somewhat diminished, such as *ras1 ras2*(Ts) strains grown at the permissive temperature or strains with attenuated A kinase activity (7). This observation reinforces the notion that A kinase activity and Yak1 activity function in opposition. The A kinase promotes growth, and the Yak1 protein tends to inhibit it. The cell balances these two activities, and the decision to continue mitotic growth is determined by which way the scale tips.

Our previous observation that reduced Yak1 activity promotes cell growth in the absence of A kinase activity argues either that it acts downstream of the A kinase or that it functions in a parallel pathway. The results presented here suggest that the latter is more likely. First, A kinase phosphorylation of Yak1 has little effect on its casein kinase activity. Certainly, this in vitro observation may reflect a more complex effect of the A kinase on Yak1 activity in vivo, as is the case for epidermal growth factor receptor-catalyzed phosphorylation of phospholipase Cγ (1). Nonetheless, the observation fails to lend support to the hypothesis that A kinase and Yak1 are in the same pathway. Second, while increased Yak1 levels coincide with diminished A kinase activity, induction of Yak1 kinase can also occur independently of changes in A kinase activity. That is, cell cycle arrest late in G_1 or early in S (events not mediated by the A kinase) also yields enhanced Yak1 levels. Further, activation of Yak1 occurs on nitrogen starvation, a process that has little effect on A kinase activity (15). Thus, we suspect that Yak1 and the A kinase constitute parallel, interdependent pathways. Phosphorylation of Yak1 by the A kinase may reflect a degree of cross-talk between these pathways.

How might Yak1 inhibit cell growth? In part, Yak1 influences expression of genes normally induced during transition to stationary phase. For instance, catalase gene expres-

sion is induced 50-fold on entry into stationary phase (3, 32). Strains with a multicopy *YAK1* plasmid show enhanced levels of catalase expression during exponential growth, and *yak1* strains fail to induce catalase following entry into stationary phase. While Yak1-mediated transcriptional regulation of a variety of genes might account for Yak1 inhibition of cell growth, Yak1 might also affect cell proliferation directly. For example, Yak1 could inactivate or repress G_1-specific cyclins encoded by *CLN1*, *CLN2*, and *CLN3* (11, 28, 40). Whatever the actual mechanism, the role of the Yak1 kinase in mediating transition to stationary phase is strikingly analogous to that of *FUS3* in mediating transition from mitotic growth to conjugation competence. *FUS3* encodes a protein kinase and is required to effect both pheromone-induced arrest of cell growth and pheromone induction of conjugation-specific genes (14).

Regulation of Yak1 activity. Consistent with the proposed role of Yak1 as an attenuator of growth, Yak1 activity increases coincident with growth arrest. This does not necessarily establish that the increase in Yak1 levels is responsible for arrest under these conditions. However, the fact that *YAK1* overexpression inhibits cell growth could suggest that the increased expression of *YAK1* under conditions of growth arrest does play a role in shutting down the cell.

The pattern of Yak1 activation is complex. First, activation can occur both by increasing the absolute amount of Yak1 protein and by increasing the specific activity of the protein. Second, activation occurs by a variety of conditions that lead to inhibition of cell growth. Thus, we cannot yet define a simple causal mechanism for activation, although several points regarding the induction pattern are worth noting.

Yak1-specific casein kinase activity can be enhanced in vivo by a mechanism independent of increased synthesis. The particular modification of Yak1 that causes its increased specific activity is unknown. Yak1 specific activity, but not Yak1 synthesis, is stimulated by nitrogen starvation, while diminished A kinase activity [in *ras*(Ts) and *cyr1*(Ts) strains] causes an increase in Yak1 levels but not apparently in its specific activity. This presents further confirmation that intracellular signalling prompted by nitrogen starvation proceeds independently of the Ras/A kinase pathway.

Increases in the absolute amount of Yak1 protein in the cell appears to be subject to cell cycle regulation. As shown here, Yak1 protein levels are induced by mutants that promote entry into G_0 (*ras*, *cyr1*, and *cdc63*; 6, 16, 17, 24) and by mutants that arrest in G_1 (*cdc28*; 26) and early in S phase (*cdc8*; 30). Levels are not increased by conditions causing arrest later in the cell cycle. While the plethora of conditions leading to increased levels may reflect multiple signals for induction, a more economical explanation would be that *YAK1* expression is cell cycle regulated. At first blush, this hypothesis seems inconsistent with the fact that little Yak1 activity is detectable during exponential growth. However, at least two models can reconcile these observations. One possibility is that the early stages of the cell cycle may simply represent a permissive state for induction, the actual signal for which might be cessation of growth. This would be similar to the situation for *HO* expression. *HO* expression is restricted to the early stage of the cell cycle, but superimposed on its cell cycle regulation, *HO* synthesis also requires that the cell be haploid and have budded at least once (5). A second possibility is that Yak1 protein levels may reflect a steady state between synthesis and decay. The early stages of the cell cycle may favor synthesis of Yak1 protein, while the later stages may favor its decay. During normal mitotic growth, little accumulation would occur. However, during arrest at an early stage in the cell cycle, the prolonged pause would allow significant accumulation of the protein. The period of accumulation of Yak1 protein is similar to that noted for G_1-specific cyclins (40). This coincidence, along with the fact that both are involved in growth control, might suggest that expression of both G_1 cyclins and Yak1 protein is regulated by a common mechanism.

ACKNOWLEDGMENTS

We thank J. Vogel for help with generating the polyclonal antibodies, D. Burke, S. Cameron, T. Toda, and A. Levine for strains and plasmids, and M. Resh and M. Igo for helpful discussions about kinase assays.

This work was supported in part by ACS institutional research grant IN-158C to S.G. and by grant CA-41086 from the National Institutes of Health to J.R.B.

REFERENCES

1. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase Cγ1, GAP, and Src to activated growth factor receptors. Science **250**:979–982.
2. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **55**:779–827.
3. **Bissinger, P. H., R. Wieser, B. Hamilton, and H. Ruis.** 1989. Control of *Saccharomyces cerevisiae* catalase T gene (*CTT1*) expression by nutrient supply via the *RAS*-cyclic AMP pathway. Mol. Cell. Biol. **9**:1309–1315.
4. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8**:17–24.
5. **Breeden, L.** 1988. Cell cycle-regulated promoters in budding yeast. Trends Genet. **4**:249–253.
6. **Brenner, C., N. Nakayama, M. Goebl, K. Tanaka, A. Toh-e, and K. Matsumoto.** 1988. *CDC33* encodes mRNA cap-binding protein eIF-4E of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:3556–3559.
7. **Cameron, S., L. Levin, M. Zoller, and M. Wigler.** 1988. cAMP-independent control of sporulation, glycogen metabolism, and heat shock resistance in *S. cerevisiae*. Cell **53**:555–566.
8. **Cannon, J., and K. Tatchell.** 1987. Characterization of *Saccharomyces cerevisiae* genes encoding subunits of cAMP-dependent protein kinase. Mol. Cell. Biol. **7**:2653–2663.
9. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapiro, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100**:293–308.
10. **Cherry, J. R., T. R. Johnson, C. Dollard, J. R. Schuster, and C. L. Denis.** 1989. Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator *ADR1*. Cell **56**:409–419.
11. **Cross, F.** 1988. *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4675–4684.
12. **Deschenes, R. J., and J. R. Broach.** 1987. Fatty acylation is important but not essential for *Saccharomyces cerevisiae RAS* function. Mol. Cell. Biol. **7**:2344–2351.
13. **Deschenes, R. J., and J. R. Broach.** 1989. The function of *RAS* genes in *Saccharomyces cerevisiae*. Adv. Cancer Res. **54**:79–138.
14. **Elion, E. A., P. L. Grisafi, and G. R. Fink.** 1990. *FUS3* encodes a $cdc2^+$/CDC28-related kinase required for the transition from mitosis into conjugation. Cell **60**:649–664.
15. **Francois, J., M. E. Villaneuva, and H. G. Hers.** 1988. The control of glycogen metabolism in yeast. 1. Interconversion *in vivo* of glycogen synthase and glycogen phosphorylase induced by glucose, a nitrogen source or uncouplers. Eur. J. Biochem.

174:551–559.
16. **Garrett, S., and J. Broach.** 1989. Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, *YAK1*, whose product may act downstream of the cAMP-dependent protein kinase. Genes Dev. **3**:1336–1348.
17. **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.
18. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241**:42–52.
19. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Jacobs, C. W., A. E. M. Adams, P. J. Azaniszlo, and J. R. Pringle.** 1988. Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **107**:1409–1426.
21. **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.
22. **Kataoka, T. D. Broek, and M. Wigler.** 1985. DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylate cyclase. Cell **43**:493–505.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Matsumoto, K., I. Uno, Y. Oshima, and T. Ishikawa.** 1982. Isolation and characterization of yeast mutants deficient in adenylate cyclase and cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **79**:2355–2359.
25. **Meluh, P. B., and M. D. Rose.** 1990. *KAR3*, a kinesin-related gene required for yeast nuclear fusion. Cell **60**:1029–1041.
26. **Mendenhall, M. D., C. A. Jones, and S. I. Reed.** 1987. Dual regulation of the yeast CDC28-p40 protein kinase complex: cell cycle, pheromone, and nutrient limitation effects. Cell **50**:927–935.
27. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
28. **Nash, R., G. Tokiwa, S. Anand, K. Erickson, and A. B. Futcher.** 1988. The *WHI1*$^+$ gene of *S. cerevisiae* tethers cell division to cell size and is a cyclin homolog. EMBO J. **7**:4335–4346.
29. **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.
30. **Pringle, J. R., and L. H. Hartwell.** 1981. The *Saccharomyces cerevisiae* cell cycle, p. 97–142. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Rubin, G. M.** 1975. Preparation of RNA and ribosomes from yeast. Methods Cell Biol. **12**:45–64.
32. **Ruis, H. (Universitat Wien).** Personal communication.
33. **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.
34. **Tatchell, K., L. C. Robinson, and M. Breitenbach.** 1985. *RAS2* of *Saccharomyces cerevisiae* is required for gluconeogenic growth and proper response to nutrient limitation. Proc. Natl. Acad. Sci. USA **82**:3785–3789.
35. **Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler.** 1985. In yeast, *RAS* proteins are controlling elements of adenylate cyclase. Cell **40**:27–36.
36. **Toda, T., S. Cameron, P. Sass, M. Zoller, and M. Wigler.** 1987. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell **50**:277–287.
37. **Tripp, M. L., R. Pinon, J. Meisenhelder, and T. Hunter.** 1986. Identification of phosphoproteins correlated with proliferation and cell cycle arrest in *Saccharomyces cerevisiae*: positive and negative regulation by cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **83**:5973–5977.
38. **Uno, I. K. Matsumoto, and T. Ishikawa.** 1982. Characterization of cyclic AMP-requiring yeast mutants altered in the regulatory subunit of protein kinase. J. Biol. Chem. **257**:14110–14115.
39. **Uno, I., K. Matsumoto, K. Adachi, and T. Ishikawa.** 1983. Genetic and biochemical evidence that trehalase is a substrate of cAMP dependent protein kinase in yeast. J. Biol. Chem. **258**:10867–10872.
40. **Wittenburg, C., K. Sugimoto, and S. 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.
41. **Wong, K.-M., and A. J. Levine.** 1989. Characterization of proteins encoded by the Epstein-Barr virus transactivator gene BMLF1. Virology **168**:101–111.
42. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468–500.