The Ras/PKA Signaling Pathway of *Saccharomyces cerevisiae* Exhibits a Functional Interaction With the Sin4p Complex of the RNA Polymerase II Holoenzyme

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ABSTRACT

Saccharomyces cerevisiae cells enter into the G_0-like resting state, stationary phase, in response to specific types of nutrient limitation. We have initiated a genetic analysis of this resting state and have identified a collection of *rye* mutants that exhibit a defective transcriptional response to nutrient deprivation. These transcriptional defects appear to disrupt the control of normal growth because the *rye* mutants are unable to enter into a normal stationary phase upon nutrient deprivation. In this study, we examined the mutants in the *rye1* complementation group and found that *rye1* mutants were also defective for stationary phase entry. Interestingly, the *RYE1* gene was found to be identical to *SIN4*, a gene that encodes a component of the yeast Mediator complex within the RNA polymerase II holoenzyme. Moreover, mutations that affected proteins within the Sin4p module of the Mediator exhibited specific genetic interactions with the Ras protein signaling pathway. For example, mutations that elevated the levels of Ras signaling, like *RAS2^{val19}*, were synthetic lethal with *sin4*. In all, our data suggest that specific proteins within the RNA polymerase II holoenzyme might be targets of signal transduction pathways that are responsible for coordinating gene expression with cell growth.

UPON nutrient deprivation, *Saccharomyces cerevisiae* cells cease mitotic division and can enter into a nondividing resting state, known as stationary phase (Werner-Washburne *et al.* 1993, 1996). During this transition, yeast cells undergo a significant change in their overall physiology that results in an elevated resistance to a number of environmental stresses, including prolonged starvation and heat shock (Werner-Washburne *et al.* 1993, 1996). In addition, the stationary phase cell exhibits a markedly reduced rate of cellular metabolism. The rate of protein translation decreases more than 200-fold, whereas the total level of mRNA is reduced at least 35-fold (Boucherie 1985; Choder 1991; Werner-Washburne *et al.* 1996). Despite this general trend, several proteins do increase in relative abundance during stationary phase and are likely responsible for many of the properties associated with this resting state (Werner-Washburne *et al.* 1993; Padilla *et al.* 1998).

The Ras protein signaling pathway appears to be a key regulator of stationary phase entry, as mutations that inactivate this pathway result in a constitutive stationary phase-like arrest (Matsumoto *et al.* 1983; Iida and Yahara 1984; Broach 1991). In contrast, elevated levels of Ras signaling prevent the acquisition of stationary phase characteristics upon nutrient deprivation (Toda *et al.* 1985). Although the *S. cerevisiae* Ras proteins have multiple effectors (Morishita *et al.* 1995; Mosch *et al.* 1996), the pathway involving cAMP and the cAMP-dependent protein kinase (PKA) is the most important for these effects on stationary phase biology (Werner-Washburne *et al.* 1993). The yeast Ras proteins, Ras1p and Ras2p, bind directly to adenylyl cyclase, Cyr1p, and stimulate the production of cAMP (Field *et al.* 1990; Suzuki *et al.* 1990). This, in turn, results in elevated levels of PKA activity and the increased phosphorylation of proteins presumably important for cell proliferation (Toda *et al.* 1987b; Broach 1991). Although several PKA substrates have been characterized, the identification of Ras/PKA targets relevant for growth control remains an area of keen interest (Reinders *et al.* 1998; Thevelein and de Winde 1999).

The entry into stationary phase is accompanied by broad changes in the patterns of gene expression that are controlled, in part, by the Ras/PKA pathway (Werner-Washburne *et al.* 1993; DeRisi *et al.* 1997). However, it is not yet known precisely how Ras activity affects the transcriptional apparatus. In *S. cerevisiae*, as in other eukaryotes, RNA polymerase (pol) II is present as a large holoenzyme complex that contains the 12-subunit polymerase, the Mediator coactivator complex, the Srb8-11 protein complex, and several general transcription factors (Koleske and Young 1995; Lee and Young 2000; Myers and Kornberg 2000). This holoenzyme is actively recruited to promoters *in vivo* as a result of specific interactions between Mediator subunits and DNA-bound transactivators (Ptashne and Gann 1997;

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Keaveney and Struhl 1998). Therefore, there are two *a priori* targets for the Ras effects on RNA pol II activity: the various transcription factors bound at the individual promoters and the regulatory proteins associated with the RNA pol II holoenzyme. Indeed, several studies suggest that the Ras pathway regulates the activity of specific transcriptional regulators, like Msn2p and Msn4p (Gorner *et al.* 1998; Thevelein and de Winde 1999). However, to date, there have been few reports of signaling pathways directly targeting components within the RNA pol II holoenzyme (Jiang *et al.* 1998; Kuchin *et al.* 2000; Chang *et al.* 2001).

We are interested in the control of stationary phase biology and have identified a collection of mutants that exhibit a defective transcriptional response to nutrient deprivation (Chang *et al.* 2001). These *rye* mutants were originally isolated on the basis of defects in the expression pattern of *YGP1*. The *YGP1* gene is induced specifically upon nutrient deprivation and this induction has been used as a marker for the ensuing entry into stationary phase (Destruelle *et al.* 1994; Riou *et al.* 1997; Chang *et al.* 2001). In the *rye* mutants, *YGP1* and related genes are expressed at an elevated level during mitotic growth (Chang *et al.* 2001). These transcriptional defects appear to disrupt the control of normal growth as the *rye* mutants are unable to enter into a normal stationary phase upon nutrient deprivation (Chang *et al.* 2001). Interestingly, three of the *RYE* genes encode Srb proteins that comprise part of the Srb complex associated with the RNA pol II holoenzyme (Chang *et al.* 2001). These observations suggested that the RNA pol II holoenzyme could be a target of signaling pathways responsible for coordinating yeast cell growth with nutrient availability.

The *rye* mutants identified in the original genetic selection defined eight complementation groups, and more than half of the mutants fell into the *rye1* group. In general, the *rye1* mutants exhibited the most severe defects in *YGP1* expression and stationary phase entry. In this report, the *RYE1* gene is characterized and shown to encode Sin4p, a component of the yeast Mediator. Thus, a second complex in the RNA pol II holoenzyme appears to be important for proper growth control in *S. cerevisiae*. In addition, *sin4* mutations exhibited specific genetic interactions with alterations that affected signaling through the Ras/PKA pathway. In all, the data suggested that Ras/PKA signaling might influence gene expression by modulating the activities of proteins associated with the RNA pol II holoenzyme.

MATERIALS AND METHODS

Growth media: Standard *Escherichia coli* growth conditions and media were used throughout this study (Miller 1972). Yeast YPAD, 5-fluoroorotic acid (5-FOA), and SC growth media were as described (Sikorski and Boeke 1991; Kaiser *et al.* 1994). YM-glucose medium refers to a yeast minimal medium containing 0.67% yeast nitrogen base (DIFCO), 2% glucose, and those growth supplements required for cell proliferation. Bromcresol purple (BCP)-sucrose medium was as described, except that 75 μg of Antimycin A_1 were top-spread onto the plates immediately before use (Robinson *et al.* 1988; Chang *et al.* 2001).

Plasmid constructions: The *YGP1-SUC2* fusion plasmid, pYGP1-SUC2, was described previously (Destruelle *et al.* 1994; Chang *et al.* 2001). The expression of this reporter is driven by *YGP1* promoter sequences present within the 350 bp immediately upstream of the *YGP1* initiation codon (Chang *et al.* 2001). The *MET3-RAS2^{val19}* plasmids were constructed as follows. The *MET3* promoter region was cloned as a 550-bp *Sal*I-*Eco*RV fragment from the pHAM8 plasmid (kindly provided by Dr. H. Mountain) into pRS403 to form pPHY440. *RAS2^{val19}* was then cloned as a 1.3-kb *Bam*HI fragment from pJW83.1 (kindly provided by Dr. J. Whistler) into pPHY440 to form pPHY446. This *RAS2^{val19}* fragment contained the coding sequences and transcriptional terminator but lacked the *RAS2* promoter. A *MET3-RAS2* plasmid, pPHY442, was constructed in a similar fashion. The *MET3-RAS2^{val19}* hybrid gene from pPHY446 was then subcloned into pRS416 to form pPHY796. The *RAS2^{val19}* plasmid, PHY453, contains the *RAS2^{val19}* allele cloned into pRS415. The pRS plasmids were described previously (Sikorski and Hieter 1989; Christianson *et al.* 1992).

Yeast strain constructions and genetic methods: The strains used in this study are listed in Table 1. Unless otherwise noted, the strains were from our lab collection or were derived during the course of this work. Standard yeast genetic methods were used for the construction of all strains (Kaiser *et al.* 1994). The isolation of the *rye* mutants was described previously (Chang *et al.* 2001). Gene disruptions were constructed with a PCR-based deletion protocol (Baudin *et al.* 1993). The *cyr1-99* allele was isolated as an extragenic suppressor of the *sin4 RAS2^{val19}* synthetic lethality. *MET3-RAS2^{val19} sin4* cells (PHY1649) were plated to YM-glucose minimal medium lacking methionine at a density of $\sim 3 \times 10^7$ cells/plate and incubated for 4 days at 30°. One suppressor was analyzed further and was found to contain an allele of *CYR1* that was designated as *cyr1-99*.

For the stationary phase experiments, yeast cells were grown in a YM-glucose minimal medium at 30°. The cultures were typically inoculated at a density of 0.1 OD_{600} units/ml. Under these conditions, the cells underwent the diauxic shift after a little more than 1 day of growth and generally entered into stationary phase after 4 days of growth (Chang *et al.* 2001). For the *MET3-RAS2^{val19}* experiments, strains carrying this inducible construct were typically grown to mid-log in YM-glucose minimal medium containing 500 μM methionine. The cells were then collected by centrifugation and resuspended in the same growth medium lacking methionine to induce expression from the *MET3* promoter.

Cloning of *RYE1/SIN4*: The *RYE1* gene was cloned by plasmid complementation of the severe flocculation phenotype exhibited by *rye1* mutants. When grown in liquid medium, the *rye1* strains formed a single cluster of cells at the bottom of the culture vessel; the medium above this cluster was almost devoid of turbidity. A yeast genomic DNA library constructed in the pSB32 plasmid was introduced into the *rye1-1* mutant, PHY1454 (Spencer *et al.* 1990). The transformed cells were separated into 16 equal aliquots and all but one was used to inoculate 6 ml of YM-glucose medium lacking leucine; the pSB32 plasmid is marked with the wild-type *LEU2* gene. The remaining aliquot was plated to solid medium to determine the transformation efficiency for the experiment. The liquid cultures were incubated overnight at 30°. A 150-μl aliquot was removed from each culture and used to inoculate a fresh 6 ml of the same medium. Following a second overnight incubation, 1 of the 15 cultures exhibited a significant degree of turbidity

TABLE 1

Yeast strains used in this study

Strain	Genotype	Alias	Source or Reference
PHY1081	*MATα his3 leu2 lys2Δ::hisG rpb1-1 trp1 ura3*		
PHY1184	*MATα his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 ura3-52* (pYGP1-SUC2)		Chang *et al.* (2001)
PHY1220	*MATα his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 ura3-52*		Chang *et al.* (2001)
PHY1222	*MAT***a** *ade2-101 his3-Δ200 leu2-3,112 suc2-Δ9 trp1-101 ura3-52*		Chang *et al.* (2001)
PHY1264	*MATα his3-Δ200 leu2-3,112 lys2-801 rye1-1/sin4 suc2-Δ9 trp1-101 ura3-52* (pYGP1-SUC2)		Chang *et al.* (2001)
PHY1447	*MATα cyr1-230 his3-11 leu2-3,112 lys2Δ::hisG suc2Δ::HIS3 trp1-1 ura3-1*		
PHY1454	*MATα his3-Δ200 leu2-3,112 lys2-801 rye1-1/sin4 suc2-Δ9 trp1-101 ura3-52*		Chang *et al.* (2001)
PHY1456	*MATα his3-Δ200 leu2-3,112 lys2-801 rye2-1/srb11 suc2-Δ9 trp1-101 ura3-52*		Chang *et al.* (2001)
PHY1459	*MATα his3-Δ200 leu2-3,112 lys2-801 rye3-1/srb9 suc2-Δ9 trp1-101 ura3-52*		Chang *et al.* (2001)
PHY1460	*MATα his3-Δ200 leu2-3,112 lys2-801 rye4-1 suc2-Δ9 trp1-101 ura3-52*		Chang *et al.* (2001)
PHY1470	*MATα his3-Δ200 leu2-3,112 lys2-801 rye5-2/srb10 suc2-Δ9 trp1-101 ura3-52*		Chang *et al.* (2001)
PHY1570	*MATα his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 tup1Δ::HIS3 ura3-52* (pYGP1-SUC2)		
PHY1575	*MATα his3-Δ200 leu2-3,112 lys2-801 sin4Δ::HIS3 suc2-Δ9 trp1-101 ura3-52* (pYGP1-SUC2)		
PHY1605	*MATα his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 ura3-52 ure2Δ::HIS3* (pYGP1-SUC2)		
PHY1647	*MATα his3-Δ200 leu2-3,112 lys2-801 rye1-1/sin4 suc2-Δ9 trp1-101 ura3-52* (pPHY442)		
PHY1649	*MATα his3-Δ200 leu2-3,112 lys2-801 rye1-1/sin4 suc2-Δ9 trp1-101 ura3-52* (pPHY446)		
PHY1669	*MATα gal11Δ::HIS3 his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 ura3-52* (pYGP1-SUC2)		
PHY1682	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1*		
PHY1720	*MAT***a** *ade2 his3 leu2 lys2 swi2::HIS3 trp1 ura3*	DY1747	D. Stillman
PHY1719	*MAT***a** *ade2 his3 leu2 lys2 sin4::TRP1 swi2::HIS3 trp1 ura3*	DY1768	Jiang and Stillman (1995)
PHY1721	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1*	DY150	Jiang and Stillman (1995)
PHY1722	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3,112 sin4::LEU2 trp1-1 ura3-1*	DY1700	D. Stillman
PHY1829	*MAT***a** *his4-917δ leu2-Δ1 lys2-173R2 rgr1-Δ2::TRP1 trp1-Δ63 ura3-52*	FY1289	Roberts and Winston (1997)
PHY1833	*MAT***a** *his3-Δ200 leu2-Δ1 lys2-173R2 snf2::LEU2 ura3-52*	FY1360	Roberts and Winston (1997)
PHY1834	*MATα his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 ura3-52* (pPHY446)		
PHY1837	*MATα his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 ura3-52* (pRS413)		
PHY2115	MATα *cyr1-230 his3-11 leu2-3,112 lys2Δ::hisG sin4::LEU2 suc2Δ::HIS3 trp1-1 ura3-1*		
PHY2121	*MATα cyr1-99 his3-Δ200 leu2-3,112 lys2-801 rye1/sin4 suc2-Δ9 trp1-101 ura3-52* (pPHY446)		
PHY2129	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3,112 med1-Δ2::HIS3 trp1-1 ura3-1*	H707	Balciunas *et al.* (1999)
PHY2130	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3,112 med2-Δ1::HIS3 trp1-1 ura3-1*	H905	Balciunas *et al.* (1999)
PHY2202	*MATα ade2 his3-Δ200 leu2-3,112 lys2-Δ201 rpb5Δ1::HIS3 ura3-52* (pRP58; *RPB5*)	WY-185	Miyao and Woychik (1998)
PHY2203	*MATα ade2 his3-Δ200 leu2-3,112 lys2-Δ201 rpb5Δ1::HIS3 ura3-52* (pRP514; *rpb5-9*)	WY-186	Miyao and Woychik (1998)
PHY2388	*MATα bcy1::LEU2 his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-101 ura3-52* (pSIN4)		
PHY2389	*MATα bcy1::LEU2 his3-Δ200 leu2-3,112 lys2-801 rye1-1/sin4 suc2-Δ9 trp1-101 ura3-52* (pSIN4)		
PHY2511	*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	YPH500	
PHY2512	*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 med9Δ::TRP1 trp1-Δ63 ura3-52*	YSJ9M	Han *et al.* (1999)
PHY2533	*MATα ade2 his3 hmr::TRP1 hrs1Δ::LEU2 leu2 trp1 ura3*	LSS59-3B	Piruat *et al.* (1997)
PHY2697	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3,112 msn2-Δ3::HIS3 msn4-1::TRP1 sin4Δ::LEU2 trp1-1 ura3-1*		

in the medium above the clumps of *rye1-1* cells. A 50-μl aliquot of this turbid culture was plated to a solid medium and the single colonies formed were analyzed. All 38 colonies analyzed gave rise to nonflocculating cultures. The library plasmids present in three of these strains were isolated and characterized further. The plasmids were found to be identical and each corrected the other phenotypes associated with the *rye1-1* mutant. Comparisons between the plasmid sequences and genomic databases were performed with the assistance of analysis programs available at the Saccharomyces Genome Database.

To ensure that the cloned gene corresponded to the *rye1* locus altered in the original mutants, a *rye1* null mutant was crossed to the original *rye1-1* strain. These *rye1*Δ*/rye1-1* diploids were sporulated and the meiotic progeny of 36 tetrads were characterized for both their growth on sucrose and their propensity to flocculate. For each tetrad, we observed that all progeny were Suc^+ and flocculated when grown in liquid culture. These data indicated that the cloned gene represented the genomic locus that was altered in the original *rye1-1* strain.

Enzyme assays: Invertase assays were performed as described, where one unit of activity is equivalent to the release of 1 nmol glucose/30 min/OD_{600} unit of cells (Johnson *et al.* 1987). β-Galactosidase assays were performed as described and the units of activity refer to the amount of *o*-nitrophenol released per minute per OD_{600} unit of cells (Ausubel *et al.* 1995). All β-galactosidase assays were performed in triplicate and the standard errors were typically <15%.

Stationary phase characteristics: Stationary phase viability assays were performed on cultures that were grown for 7 to 10 days in YM-glucose medium. Cells were collected by centrifugation and resuspended in distilled water at a concentration of 1 OD_{600} unit/ml. The suspensions were subjected to a series of fivefold dilutions and 200 μl of each suspension was placed into a well of a microtitre plate. These suspensions were then plated with a 48-prong replicating block to YPAD medium. The plates were incubated for 3 days at 30° and the relative number of survivors was determined for each strain analyzed.

The heat-shock sensitivity of the appropriate cultures was tested after 4 days of growth in minimal medium at 30°. For these assays, 200-μl aliquots of the cultures were placed into a microcentrifuge tube and incubated at 50° for 30 min. Dilutions of the cultures were plated to YPAD medium before the initiation of the heat shock and at 10-min intervals thereafter. These plates were then incubated for 3 days at 30°. The relative survival rate was determined by comparing the number of colonies formed by the cultures after heat shock to the number formed by the original culture.

RNA analyses: Total RNA was prepared from yeast cells by a hot phenol extraction method described previously (Ausubel *et al.* 1995). For Northern analyses, 20 μg of total RNA per lane was loaded onto a formaldehyde-agarose gel and subjected to electrophoretic separation. The gel was blotted to nylon membranes that were then hybridized with the appropriate ^{32}P-labeled probes (Ausubel *et al.* 1995). Typically, these probes were 0.7- to 1.0-kb PCR fragments that were prepared with the oligolabeling kit (Amersham). To ensure uniform loading for the stationary phase RNA experiments, rRNA levels were assessed visually after staining the gel with ethidium bromide.

RESULTS

***RYE1/SIN4* function was required for the regulation of *YGP1*:** All of the *rye1* mutants tested exhibited an elevated level of *YGP1* expression during the log phase of growth (Figure 1; data not shown). For these assays, the cells contained a *YGP1-SUC2* hybrid reporter that was used in the original *rye* mutant selection (Chang *et al.* 2001). The *SUC2* gene encodes invertase, an enzyme that is necessary for yeast cell growth on sucrose (Johnston and Carlson 1992). In wild-type cells, this reporter was expressed at very low levels during log phase growth and was induced >150-fold upon entry into stationary phase (Figure 1A; see Destruelle *et al.* 1994; Chang *et al.* 2001). This induction was relatively specific to stationary phase entry as no significant increase in expression was observed in response to a variety of other stresses, including heat shock and increased osmolarity (Chang *et al.* 2001). In the *rye1-1* mutant, the log phase level of this *YGP1* reporter was 57 times higher than that seen in an isogenic wild-type strain (Figure 1, A and B). This elevated expression of *YGP1-SUC2* allowed *rye1* mutants to grow on sucrose-containing media, whereas *RYE* strains were phenotypically Suc^- (Figure 1C). The *rye1* mutations also resulted in elevated levels of expression from the endogenous *YGP1* locus (Chang *et al.* 2001). Therefore, the *RYE1* gene was required for the normal regulation of *YGP1* expression.

The *rye1* mutants exhibited a number of additional phenotypes, including temperature-sensitive (*ts*) growth defects, a severe propensity to flocculate, and a partial inositol auxotrophy. The wild-type *RYE1* locus was cloned by complementation of this flocculation defect (see materials and methods) and the identified gene was found to complement all of the phenotypes ascribed to *rye1* mutations. Interestingly, DNA sequence analysis revealed that *RYE1* was identical to the previously identified gene, *SIN4*. The *SIN4* gene encodes a protein that functions as both a positive and negative regulator of RNA pol II transcription (Jiang and Stillman 1992; Chen *et al.* 1993; Jiang *et al.* 1995). Previous work has shown that Sin4p is associated with the RNA pol II holoenzyme and that Sin4p may exert its effects by altering chromatin structure (Jiang and Stillman 1992; Li *et al.* 1995). We constructed a null allele of the *RYE1/SIN4* locus and found that this deletion resulted in a spectrum of phenotypes similar to that observed with other *rye1* alleles (Figure 1; data not shown). Since the *SIN4* gene has been extensively characterized, we refer to the *RYE1/SIN4* locus as *SIN4* for the remainder of this report.

Sin4p is part of a complex within the Mediator that contains Gal11p, Hrs1p, and Med2p (Li *et al.* 1995; Myers *et al.* 1999). Mutations in the genes encoding these proteins typically have overlapping but distinct defects in RNA pol II transcription (Myers and Kornberg 2000). Therefore, the expression of the *YGP1-SUC2* reporter was examined in *gal11*Δ, *hrs1*Δ, and *med2*Δ mutants. We found that the reporter levels were significantly elevated in *gal11*Δ and *hrs1*Δ mutants but not in *med2*Δ mutants (Figure 2A). In addition, reporter levels were not elevated in cells lacking Med1p, a Media-

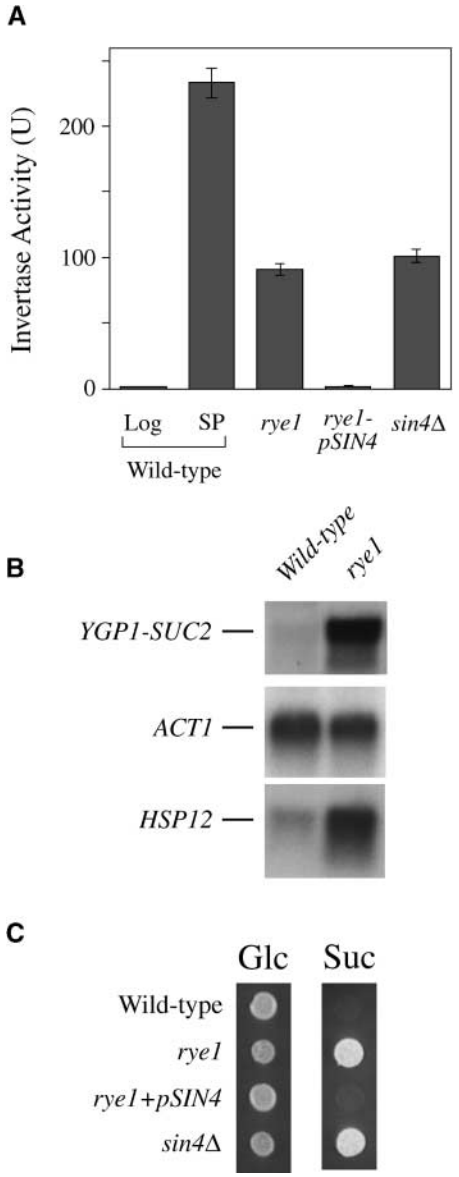

FIGURE 1.—*RYE1/SIN4* was required for the proper regulation of the *YGP1-SUC2* reporter and related genes. (A) The *YGP1* expression defect in *rye1* mutants was complemented by the presence of a *SIN4* plasmid. Yeast strains carrying the pYGP1-SUC2 reporter were grown to mid-log phase at 30° in YM-glucose minimal medium and assayed for invertase activity. The strains analyzed were wild type (PHY1184), *rye1* (PHY1264), and *sin4*Δ (PHY1575). The *rye1* strain was analyzed with and without the pSIN4 plasmid. The level of invertase activity found in wild-type stationary phase cultures that were grown for 7 days in minimal medium is shown for comparison (SP). Each value represents the average of three independent experiments. (B) mRNA levels in *rye1* mutants. The steady-state levels of *YGP1-SUC2*, *HSP12*, and *ACT1* mRNA in mid-log cultures of wild-type (PHY1184) and *rye1* (PHY1264) strains were measured by Northern RNA blot analysis. Twenty micrograms of total RNA were loaded for each sample. (C) The *sin4*Δ mutant exhibited a Rye$^-$ growth phenotype. The indicated strains were grown for 2 to 3 days at 30° on either YPAD (Glc) or BCP-sucrose (Suc) media. The strains analyzed were wild type (PHY1184), *rye1* (PHY1264), and *sin4*Δ (PHY1575).

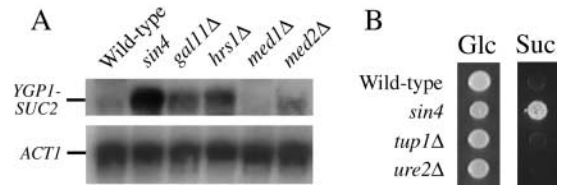

FIGURE 2.—Other components of the Sin4p module of the Mediator complex were required for proper regulation of *YGP1* expression. (A) Several components of the Sin4p module of the Mediator were required for the normal regulation of the *YGP1-SUC2* reporter. The steady-state levels of *YGP1-SUC2* and *ACT1* mRNA in mid-log cultures of the indicated strains were measured by Northern RNA blot analysis. Twenty micrograms of total RNA were loaded for each sample. The strains analyzed were wild type (PHY1220), *sin4* (PHY1455), *gal11*Δ (PHY1669), *hrs1*Δ (PHY2533), *med1*Δ (PHY2129), and *med2*Δ (PHY2130). Each strain was carrying the pYGP1-SUC2 plasmid. Note that for each mutant, the reporter levels were compared to those in an isogenic wild-type control. (B) Neither Tup1p nor Ure2p was required for the repression of the *YGP1-SUC2* reporter. The indicated strains carrying the pYGP1-SUC2 reporter plasmid were grown for 2 to 3 days at 30° on either YPAD (Glc) or BCP-sucrose (Suc) media. The strains analyzed were wild type (PHY1184), *sin4* (PHY1264), *tup1*Δ (PHY1570), and *ure2*Δ (PHY1605).

tor component not thought to be associated with the Sin4p module (Figure 2A). Finally, strains defective in both the Srb complex and the Sin4p module, such as *sin4 srb10* mutants, did not exhibit any additional *YGP1* expression defects (data not shown). Thus, as observed with other promoters, individual Mediator components appeared to make distinct contributions to the normal expression pattern of the *YGP1-SUC2* reporter (HAN *et al.* 1999; MALIK and ROEDER 2000; MYERS and KORNBERG 2000).

The above data indicated that Sin4p and associated proteins were required for the efficient repression of *YGP1* during the log phase of growth. We also examined the roles of two additional transcriptional regulators, Tup1p and Ure2p, in the regulation of *YGP1*. Tup1p is a negative regulator of the transcription of many genes in yeast, and mutations in *SIN4* often weaken Tup1-mediated repression (WAHI and JOHNSON 1995; FRIESEN *et al.* 1998; LEE *et al.* 2000). Ure2p is required for the full repression of many genes important for N_2 metabolism (MAGASANIK 1992). These genes include a family of asparaginase enzymes that exhibit sequence similarity to Ygp1p (BON *et al.* 1997). We found that deletion of either *TUP1* or *URE2* did not result in a Rye$^-$ phenotype, and thus neither gene product was required for the repression of the *YGP1-SUC2* reporter (Figure 2B).

***rye1/sin4* mutants did not enter a normal stationary phase upon nutrient deprivation:** The entry into stationary phase appears to involve a tightly regulated program of gene expression (CHODER 1991; WERNER-WASHBURNE *et al.* 1993, 1996). Whereas some genes are induced at the earliest stages of nutrient deprivation, the expression of others increases at later stages. Moreover,

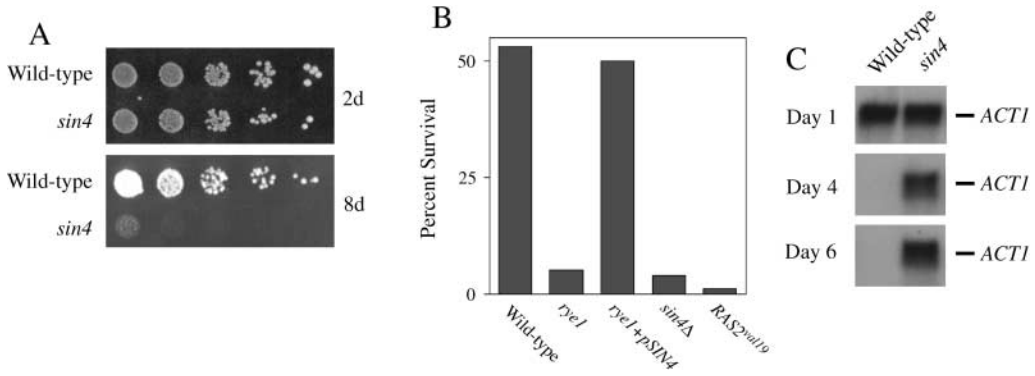

FIGURE 3.—*SIN4* function was required for the entry into a normal stationary phase. (A) The *rye1* mutants exhibited decreased viability following an extended period of nutrient deprivation. Wild-type (PHY1184) and *rye1* (PHY1264) strains were grown at 30° for either 2 or 8 days in YM-glucose minimal medium. Cells from these cultures were collected by centrifugation, washed, and resuspended in distilled water at a concentration of 1 OD_{600} unit/ml. Fivefold serial dilutions of these suspensions were then plated to YPAD medium and incubated for 3 days at 30°. The number of colonies formed was a measure of the number of survivors in the original cultures. (B) Stationary phase cultures of *sin4/rye1* mutants were sensitive to a brief heat shock. The indicated strains were grown at 30° for 4 days in YM-glucose minimal medium and then subjected to a 50° heat shock for 20 min. The percentage of cells surviving this treatment are shown. The strains analyzed were wild type (PHY1184), *rye1* (PHY1264), and *sin4*Δ (PHY1575). The $RAS2^{val19}$ control is the wild-type strain (PHY1220) carrying a plasmid with the dominant $RAS2^{val19}$ allele. The standard errors were typically <10% and each value represents the average of three independent experiments. (C) *sin4* mutants exhibited defects in the stationary phase repression of *ACT1*. The steady-state level of *ACT1* mRNA in stationary phase cultures of wild-type (PHY1184) and *sin4* (PHY1264) strains was measured by Northern blot analysis. Total RNA was prepared from cultures grown for 1, 4, or 6 days at 30° in minimal medium. Twenty micrograms of total RNA was loaded for each sample. As a control for loading, rRNA levels were assessed by visual inspection after staining the gel with ethidium bromide.

disruptions of this transcriptional program result in a failure to enter into a normal stationary phase (CHANG *et al.* 2001). The above data with *YGP1* clearly indicated that such a disruption might be associated with a loss of *SIN4* function. Indeed, *sin4* mutations also affected the expression patterns of other genes normally expressed during nutrient limitation. For example, the *CTT1* and *HSP12* genes are normally expressed at very low levels during log phase but are significantly induced during the diauxic shift (PRAEKELT and MEACOCK 1990; DERISI *et al.* 1997). In *sin4* mutants, the log phase level of each of these mRNAs was elevated more than fivefold (Figure 1B; data not shown). Therefore, we tested whether *sin4* mutants were able to enter into a normal stationary phase upon nutrient deprivation.

One of the hallmarks of a stationary phase yeast cell is the ability to survive for extended periods of time under nutrient-limiting conditions (WERNER-WASHBURNE *et al.* 1993). In contrast, mutants that fail to enter into a normal stationary phase rapidly lose viability upon nutrient deprivation (CANNON and TATCHELL 1987; TODA *et al.* 1987a; WERNER-WASHBURNE *et al.* 1993). Therefore, we assessed the relative number of survivors in stationary phase cultures of wild-type and *sin4* strains. As expected, wild-type cells remained viable after 8 days of growth in minimal medium (Figure 3A). The number of survivors after 8 days of growth was not significantly different than that observed after 2 days. In contrast, after 8 days, the *sin4* cultures had at least 200-fold fewer survivors than the wild type (Figure 3A). Thus, *sin4* mutants were unable to survive a prolonged period of nutrient limitation.

The response of *sin4* mutants to nutrient deprivation was examined further by assessing two additional properties normally associated with the stationary phase of growth. Stationary phase cells generally exhibit an elevated resistance to a number of environmental stresses, including heat shock (WERNER-WASHBURNE *et al.* 1993). Therefore, stationary phase cultures of wild-type and *sin4* cells were subjected to a 50° heat shock for 20 min. We found that the *sin4* cultures were significantly more sensitive than wild type to this heat-shock regimen (Figure 3B). Finally, we examined the general decrease in RNA pol II transcription that occurs upon stationary phase entry. In general, stationary phase levels of most mRNAs are significantly lower than that observed during log phase growth (CHODER 1991). This repression phenomenon has been best characterized for the *ACT1* locus and hence *ACT1* mRNA levels were assessed in the *sin4* cultures. Once again, the *sin4* mutants did not respond normally to nutrient deprivation and contained significantly elevated levels of *ACT1* mRNA after 6 days of growth (Figure 3C). Altogether, these data indicated that Sin4p was required for stationary phase entry in *S. cerevisiae.*

Mutations that elevate the level of Ras signaling were synthetically lethal with *sin4*: The Ras/PKA signaling pathway appears to negatively regulate *YGP1* expression because decreased levels of Ras activity result in increased levels of the *YGP1-SUC2* reporter (CHANG *et al.* 2001). Therefore, the *rye* mutants could identify targets of the Ras pathway that are important for this transcriptional control. This possibility was tested by asking whether elevated levels of Ras signaling would suppress *rye* mutant phenotypes. Indeed, the presence of a dominant hyperactive allele of *RAS2*, known as $RAS2^{val19}$ (KATAOKA *et al.* 1984), was able to suppress the *YGP1*

misexpression phenotype associated with *rye4* mutants (data not shown). However, in *sin4* cells, the presence of *RAS2*^{val19} instead resulted in a severe synthetic growth defect.

This unexpected growth defect associated with *sin4 RAS2*^{val19} mutants was demonstrated in three independent assays. The first was a transformation-based assay where we found that a plasmid containing *RAS2*^{val19} could not be stably introduced into *sin4* cells. The frequency of transformation with *sin4* mutants for plasmids with *RAS2*^{val19} was 5000-fold less than that for control plasmids. For the second assay, an inducible allele of *RAS2*^{val19} was constructed by placing the *RAS2*^{val19} coding sequences under the control of the yeast *MET3* promoter. This promoter is active when cells are grown in media lacking methionine and is repressed by the presence of methionine in the growth medium (Cherest *et al.* 1987; Mountain *et al.* 1991). The introduction of this *MET3-RAS2*^{val19} construct into *sin4* cells resulted in a severe growth defect specifically in media lacking methionine (Figure 4A). Note that the growth rates of the *sin4* and *RAS2*^{val19} single mutants were very similar to that of the isogenic wild-type control (Figure 4A). Finally, *sin4 RAS2*^{val19} mutants were unable to lose a plasmid that contained the wild-type *SIN4* locus (Figure 4B). In contrast, this *SIN4* plasmid was readily lost from either wild-type or both single mutant cells (Figure 4B). A similar synthetic growth defect with *RAS2*^{val19} was exhibited by all other *sin4* alleles, including the null. These growth defects were not specific to the genetic background used for these studies because *sin4* mutations in five different backgrounds were all found to be sensitive to *RAS2*^{val19}. Thus, elevated levels of Ras signaling caused a severe growth defect in *sin4* cells.

In the above experiments, most of the *sin4* cells were likely in stationary phase before they were tested for growth with *RAS2*^{val19}. Therefore, it was a formal possibility that the double mutant growth defect was specific to stationary phase cells and that our assays were measuring a defect in stationary phase exit. However, this possibility was ruled out since the induction of *RAS2*^{val19} in log phase *sin4* cultures also resulted in a severe growth arrest (Figure 5A). This arrest occurred with very rapid kinetics as the *sin4* cells did not undergo even a single round of division following the induction of *RAS2*^{val19} expression. Moreover, the elevated levels of Ras signaling caused the *sin4* cells to rapidly lose viability; $<0.01\%$ of the *sin4* cells remained viable 2 hr after the induction of *RAS2*^{val19} (Figure 5B). Therefore, elevated levels of Ras signaling caused a rapid growth arrest and subsequent cell death specifically in *sin4* mutants.

Since Sin4p is involved in transcriptional regulation, we tested whether the *sin4 RAS2*^{val19} lethality was due to a general defect in mRNA production. For this analysis, the steady-state levels of multiple mRNAs were assessed in wild-type and *sin4* cells at 0, 2, and 4 hr after induction of *RAS2*^{val19} expression. At these latter two time points, the *sin4* cultures contained very few, if any, viable cells (Figure 5B). Nonetheless, the levels of each of the mRNAs tested remained unchanged throughout the course of this experiment (Figure 6). More importantly, the levels in wild-type and *sin4* cells were essentially identical (Figure 6). Thus, the loss of *sin4 RAS2*^{val19} viability was not correlated with a global defect in RNA pol II transcription. Instead, the observed synthetic lethality might be due to transcriptional defects at a subset of essential genes.

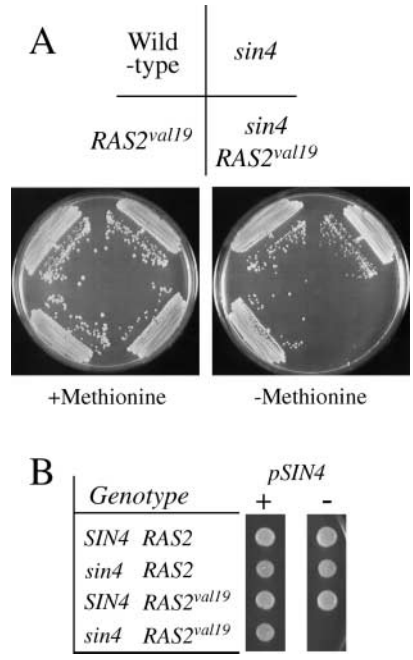

Figure 4.—The presence of *RAS2*^{val19} resulted in a severe synthetic growth defect specifically in *sin4* mutants. (A) *sin4 RAS2*^{val19} double mutants exhibited a synthetic growth defect. The indicated strains were grown on YM-glucose minimal media that contained either 0 or 500 μM methionine for 3 days at 30°. The strains analyzed were wild type (PHY1837), *sin4* (PHY1647), *RAS2*^{val19} (PHY1834), and *sin4 RAS2*^{val19} (PHY1649). The *RAS2*^{val19} strains contained *RAS2*^{val19} under the control of the methionine-repressible promoter from the yeast *MET3* gene. Therefore, *RAS2*^{val19} was expressed only on media lacking methionine. (B) Growth of *RAS2*^{val19} *sin4* double mutants required the presence of the pSIN4 plasmid. The indicated strains containing the pSIN4 plasmid were grown for 3 days at 30° on either YM-glucose minimal (+pSIN4) or 5-FOA (−pSIN4) media. The 5-FOA medium selects against the *URA3*-marked pSIN4 plasmid; therefore, only those strains that can be cured of this plasmid will exhibit growth on 5-FOA medium. The strains analyzed were wild type (PHY1220 with pRS415), *sin4* (PHY1454 with pRS415), *RAS2*^{val19} (PHY1220 with pPHY453), and *sin4 RAS2*^{val19} (PHY1454 with pPHY453).

The *RAS2*^{val19} *sin4* synthetic lethality required the cAMP/PKA pathway: The *S. cerevisiae* Ras proteins have been shown to function through at least three different effectors: the cAMP/PKA pathway, a MAP kinase pathway important for pseudohyphal growth, and a poorly defined third effector that is required for the exit from

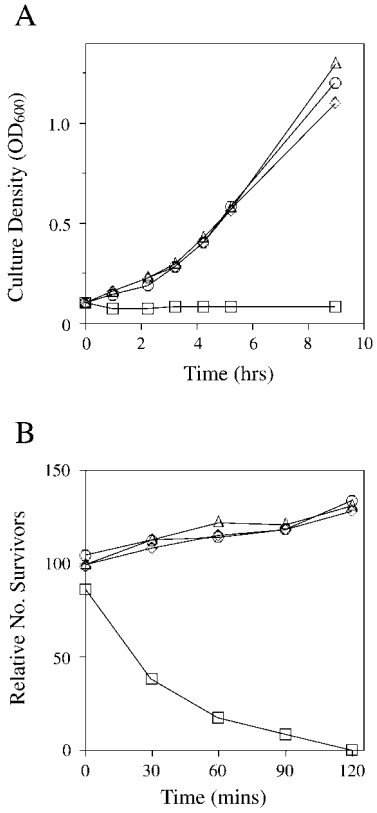

FIGURE 5.—Increased Ras signaling resulted in a rapid growth arrest and subsequent loss of viability in *sin4* cells. (A) The expression of *RAS2*^{val19} in log phase *sin4* cells resulted in a rapid growth arrest. Yeast strains were grown to mid-log in YM-glucose minimal medium containing 500 μM methionine. The cells were then transferred to media lacking methionine to induce expression from the *MET3-RAS2*^{val19} construct. The subsequent growth of the culture in this medium was monitored by measuring the optical density of the culture at 600 nm. The strains analyzed were wild type (PHY1837; △), *sin4* (PHY1647; ○), *RAS2*^{val19} (PHY1834; ◇), and *sin4 RAS2*^{val19} (PHY1649; □). All growth was carried out at 30°. (B) The expression of *RAS2*^{val19} resulted in a rapid cell death in *sin4* mutants. Yeast strains were grown as described in A. Following the shift to media lacking methionine, cells were collected at the indicated intervals, diluted in water, and plated to YPAD media. These plates were incubated for 3 days at 30°. The number of colonies present was a measure of the number of viable cells in the original cultures. The relative number of survivors was determined by calculating the number of colonies formed per OD_{600} unit and normalizing these values to that obtained for the wild-type strain at time zero.

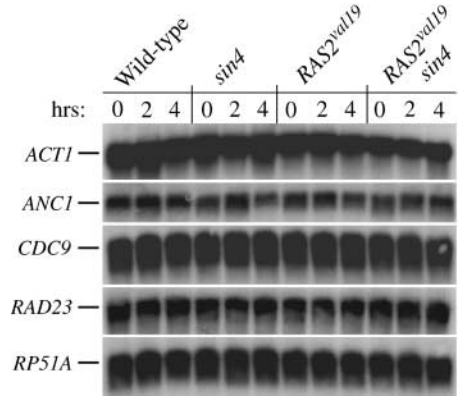

FIGURE 6.—*RAS2*^{val19} *sin4* double mutants did not exhibit a global defect in mRNA production. Yeast strains were grown at 30° to mid-log in YM-glucose minimal medium containing 500 μM methionine. The cells were then transferred to media lacking methionine to induce the expression from the *MET3-RAS2*^{val19} construct. Cells were collected at 0, 2, and 4 hr after the shift and total RNA was prepared as described in MATERIALS AND METHODS. The steady-state levels of *ACT1*, *ANC1*, *CDC9*, *RAD23*, and *RP51A* mRNA were then measured by Northern RNA blot analysis. Twenty micrograms of total RNA were loaded for each sample. The strains analyzed were wild type (PHY1837), *sin4* (PHY1647), *RAS2*^{val19} (PHY1834), and *sin4 RAS2*^{val19} (PHY1649).

mitosis (GIBBS and MARSHALL 1989; MORISHITA *et al.* 1995; MOSCH *et al.* 1996). The importance of the PKA pathway for the *sin4 RAS2*^{val19} lethality was indicated by two independent lines of investigation. In the first, *sin4* mutations were combined with null alleles of *BCY1* and the viability of the double mutants was tested. *BCY1* encodes the inhibitory subunit of PKA, and deletion of *BCY1* results in elevated levels of PKA activity without affecting the Ras proteins. In this experiment, the *BCY1* locus was deleted in *sin4* cells that carried the wild-type *SIN4* gene on a plasmid. The resulting *sin4 bcy1*Δ double mutant was unable to lose the *SIN4* plasmid indicating that *sin4* mutations are synthetically lethal with alterations that elevate the levels of PKA activity (Figure 7A).

The second strategy used to test the importance of the Ras/PKA pathway involved lowering the level of cAMP produced in *sin4 RAS2*^{val19} cells. This was accomplished by introducing two different *cyr1* alleles, *cyr1-230* and *cyr1-99*, into the above double mutant. *CYR1* encodes the yeast adenylyl cyclase, and *cyr1* mutations would be expected to lower the cellular levels of cAMP and thus PKA activity (MATSUMOTO *et al.* 1982). Indeed, both of these *cyr1* alleles were found to restore near normal growth rates to the *sin4 RAS2*^{val19} double mutant (Figure 7B). Therefore, the lethal effects of *RAS2*^{val19} in *sin4* mutants were due to the elevated levels of PKA present.

Although PKA is likely to phosphorylate a number of substrates important for *S. cerevisiae* growth, few of these potential targets have been identified (REINDERS *et al.* 1998; THEVELEIN and DE WINDE 1999). However, recent data indicate that two related transcription factors, Msn2p and Msn4p, might be targets of the Ras/PKA signaling pathway (GORNER *et al.* 1998; SMITH *et al.* 1998). Msn2p and Msn4p are required for the transcription of a number of genes induced during the cellular response to environmental stress (MARCHLER *et al.* 1993; SCHMITT and MCENTEE 1996). Interestingly, the deletion of both *MSN2* and *MSN4* suppresses the otherwise lethal loss of all three genes encoding catalytic subunits of the yeast PKA (SMITH *et al.* 1998). These observations led to the proposition that Ras/PKA activity was negatively regulat-

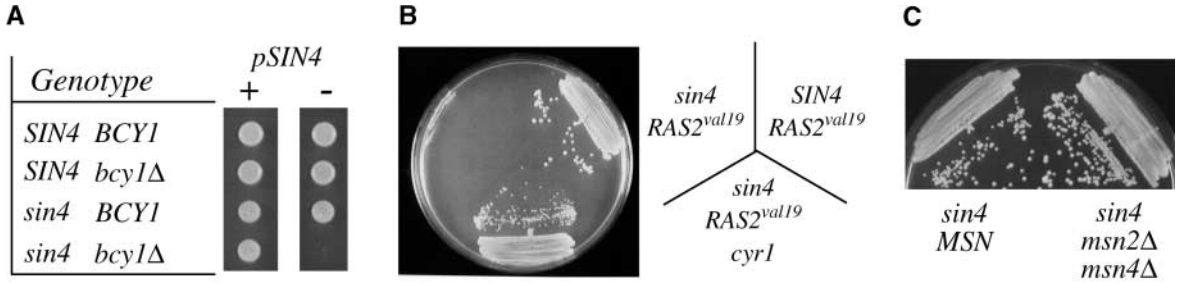

FIGURE 7.—The *RAS2*^{val19} *sin4* synthetic lethality required the cAMP/PKA signaling pathway. (A) *sin4 bcy1* double mutants exhibited a synthetic lethal growth defect. The indicated strains containing the pSIN4 plasmid were grown for 3 days at 30° on either YM-glucose minimal (+pSIN4) or 5-FOA (−pSIN4) media. The 5-FOA medium selects against the *URA3*-marked pSIN4 plasmid; therefore, only those strains that can be cured of this plasmid will exhibit growth on 5-FOA medium. The strains analyzed were wild type (PHY1220), *sin4* (PHY1454), *bcy1* (PHY2388), and *sin4 bcy1* (PHY2389). (B) The *RAS2*^{val19} *sin4* synthetic lethality was suppressed by the presence of *cyr1* mutations that lower the level of Ras/PKA signaling. The indicated strains all contained the *MET3-RAS2*^{val19} construct (pPHY446) and were grown on YM-glucose minimal medium lacking methionine for 3 days at 30°. The strains analyzed were *RAS2*^{val19} (PHY1834), *sin4 RAS2*^{val19} (PHY1649), and *sin4 cyr1-99 RAS2*^{val19} (PHY2121). (C) The *sin4 msn2*Δ *msn4*Δ triple mutant was viable and exhibited a normal growth rate. *sin4* (PHY1722) and *sin4 msn2*Δ *msn4*Δ (PHY2697) strains were incubated on YPAD plates for 2 days at 30°.

ing Msn2p/Msn4p function. Therefore, we tested whether the *sin4 RAS2*^{val19} lethality observed here was mediated by Msn2p and/or Msn4p. If these two Msn proteins are the primary target of Ras signaling, deletion of both *MSN2* and *MSN4* should also be synthetic lethal with *sin4*. However, the triple *msn2 msn4 sin4* mutant was viable and exhibited a wild-type growth rate (Figure 7C). In addition, the loss of *MSN2* and *MSN4* did not suppress the *sin4 RAS2*^{val19} lethality (data not shown). Therefore, the Ras effects on *sin4* growth appear to involve PKA targets other than these two transcription factors.

Mutations that affect the Sin4p module of the Mediator were synthetic lethal with *RAS2*^{val19}: Our data indicate that *sin4* mutants have several phenotypes in common with mutants that possess high levels of Ras signaling activity. In addition to the stationary phase defects described above, *sin4* and *RAS2*^{val19} mutants exhibited similar *ts* growth defects, inositol auxotrophy, and flocculation phenotypes. One potential explanation for these similarities is that Sin4p is a negative regulator of some aspect of Ras signaling. However, several observations indicated that this possibility was unlikely. First, the levels of both Ras proteins, and of intracellular cAMP, were very similar in wild-type and *sin4* cells (data not shown). In addition, *sin4* mutations were not able to suppress the growth defects associated with mutations that lower the level of Ras signaling, such as *cdc25-1*, *ras2-23*, and *cyr1-230* (data not shown). Therefore, Sin4p did not appear to be a negative regulator of Ras protein expression or signaling activity.

To further examine the interaction between Ras/PKA signaling activity and Sin4p, we tested whether other mutations affecting the Mediator and RNA pol II were influenced by the presence of elevated levels of Ras signaling. These experiments indicated that the synthetic lethality with *RAS2*^{val19} was relatively specific to mutations affecting the Sin4p-containing module of the Mediator. Mutations in *GAL11*, *MED2*, *HRS1*, and *RGR1* were all synthetically lethal with *RAS2*^{val19} (Figure 8). *RGR1* encodes a protein that is thought to link the Sin4p module to the remainder of the Mediator complex (LI *et al.* 1995). In contrast, mutations that affected other Mediator components (*med1* and *med9*), the Srb complex (*srb9*, *srb10*, and *srb11*), the Snf/Swi chromatin remodeling complex (*swi2*), or RNA pol II (*rpb1* and *rpb5*) were relatively insensitive to changes in Ras signaling activity (Figure 8). *SNF2/SWI2* was tested because *sin4* mutations have been shown to suppress transcriptional defects associated with the loss of this gene (JIANG and STILLMAN 1992; SONG *et al.* 1996). In all, these genetic data suggest the existence of a functional interaction between the Ras/PKA signaling pathway and the Sin4p module of the Mediator complex.

Some *sin4* phenotypes were suppressed by mutations that lower Ras signaling activity: The presence of elevated levels of Ras signaling activity had a very dramatic effect on cells lacking the *SIN4* gene. Interestingly, we found that lowering Ras/PKA activity also influenced specific *sin4* phenotypes. For example, the presence of a *cyr1* mutation suppressed the flocculation phenotype associated with particular *sin4* mutants (Figure 9). Moreover, *cyr1* mutations also suppressed the *CTS1* expression defect caused by the loss of Sin4p. *CTS1* encodes an endochitinase, and *CTS1* expression is decreased 2.5- to 3-fold in *sin4* mutants (KURANDA and ROBBINS 1991; JIANG *et al.* 1995). This *CTS1* expression defect was corrected in *cyr1 sin4* double mutants (Figure 9). Thus, both raising and lowering Ras/PKA signaling activity had profound effects on *sin4* phenotypes.

Previous work has shown that *sin4* mutations affect the expression of a number of genes in yeast (JIANG

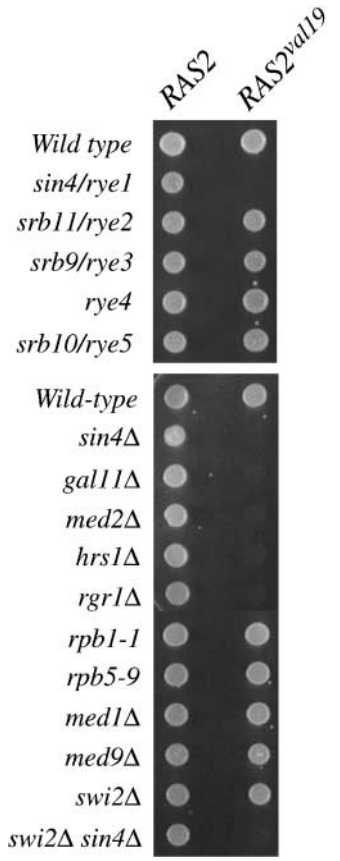

FIGURE 8.—Mutations that affected the Sin4p module of the RNA pol II holoenzyme exhibited a synthetic lethal interaction with *RAS2*^{val19}. Strains with the indicated genotype were transformed with either a control vector, pRS416 (*RAS2*), or a plasmid containing the *MET3-RAS2*^{val19} construct, pPHY796 (*RAS2*^{val19}). The strains were plated to YM-glucose minimal media lacking methionine and incubated for 2 to 3 days at 30°. (Top) The *RAS2*^{val19} growth defect was specific to *sin4/rye1* mutants. The strains analyzed were wild type (PHY1220), *sin4/rye1* (PHY1454), *srb11/rye2* (PHY1456), *srb9/rye3* (PHY1459), *rye4* (PHY1469), and *srb10/rye5* (PHY1470). (Bottom) The growth effects of *RAS2*^{val19} on various mutations affecting RNA pol II activity. The strains analyzed were wild type (PHY1220), *sin4*Δ (PHY1575), *gal11*Δ (PHY1669), *med2*Δ (PHY2130), *hrs1* (PHY2533), *rgr1*Δ (PHY1829), *rpb1-1* (PHY1081), *rpb5-9* (PHY-2203), *med1*Δ (PHY2129), *med9*Δ (PHY2512), *swi2*Δ (PHY1719), and *swi2 sin4* (PHY1720).

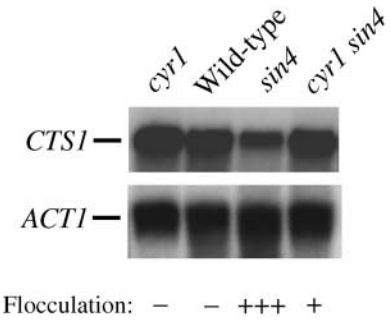

FIGURE 9.—Decreased signaling through the Ras/PKA pathway suppressed the flocculation and *CTS1* transcription defects associated with *sin4* mutants. The steady-state levels of *CTS1* and *ACT1* mRNA in mid-log cultures of the indicated strains were measured by Northern RNA blot analysis. Twenty micrograms of total RNA were loaded for each sample. The strains analyzed were wild type (PHY1721), *sin4* (PHY1722), *cyr1* (PHY1447), and *cyr1 sin4* (PHY2115). The relative degree of flocculation observed for the following strains is indicated below the RNA blot: wild type (PHY1220), *sin4* (PHY1454), *cyr1* (PHY2121 with pSIN4), and *cyr1 sin4* (PHY2121). These strains were grown to mid-log in YM-glucose minimal medium containing 500 μM methionine.

and STILLMAN 1992; CHEN *et al.* 1993; COVITZ *et al.* 1994; WAHI and JOHNSON 1995). Therefore, we tested whether lowering Ras signaling activity would suppress other transcriptional defects associated with *sin4* mutants. For these experiments, three different reporter genes were analyzed in wild-type, *sin4*, *cyr1*, and *cyr1 sin4* strains. The expression of each of these reporter genes, *PHO5::lacZ*, *HIS4::lacZ*, and *Ty1::lacZ*, is altered in *sin4* mutants (JIANG and STILLMAN 1992, 1995). However, in contrast to the above results with *CTS1*, we found that *cyr1* mutations had no significant effect on the *sin4* defects associated with these three reporters (data not shown). Therefore, Ras/PKA signaling appears to affect only specific phenotypes associated with the loss of *SIN4* function.

DISCUSSION

We are interested in understanding the mechanisms regulating stationary phase biology in *S. cerevisiae*. To this end, we have identified a collection of *rye* mutants that exhibit defects in the transcriptional response to nutrient deprivation (CHANG *et al.* 2001). These transcription defects appear to disrupt yeast cell growth as all of the *rye* mutants characterized to date are unable to enter into a normal stationary phase. In this report, we examined the *rye1* mutants and found that these mutants also exhibited multiple stationary phase defects. The *RYE1* gene was cloned and was found to encode Sin4p, a component of the yeast Mediator complex associated with the RNA pol II holoenzyme. Sin4p is part of a subcomplex that also contains Gal11p, Hrs1p, and Med2p (LI *et al.* 1995; MYERS *et al.* 1999). Mutations that inactivated Gal11p and Hrs1p also resulted in a Rye$^-$ phenotype, indicating that this Sin4p module is generally important for the regulation of genes like *YGP1*. Finally, a specific genetic interaction was observed between this Sin4p module of the Mediator and the Ras/PKA signaling pathway. We found that elevated levels of Ras/PKA signaling resulted in a synthetic lethality specifically with mutations that compromised the Sin4p module. Mutations affecting other components of the Mediator or RNA pol II itself were not significantly affected by changes in Ras/PKA signaling activity. Altogether, the data presented here and in a previous study indicate that transcriptional regulators within the RNA

pol II holoenzyme are required for the normal control of cell growth in *S. cerevisiae* (Chang *et al.* 2001).

Two distinct complexes within the RNA pol II holoenzyme are required for proper stationary phase entry: Wild-type cells respond to nutrient deprivation by undergoing an orderly series of changes in gene expression (Choder 1991; DeRisi *et al.* 1997). Our studies with the *rye* mutants suggest that the proper execution of this transcriptional program is necessary for stationary phase entry. Mutations in *SIN4* and the other *RYE* genes disrupt the transcriptional response to nutrient deprivation and prevent cells from entering into a normal stationary phase (Chang *et al.* 2001). In *rye* mutants, genes normally induced upon nutrient limitation, such as *YGP1*, are instead expressed at elevated levels during log phase growth (Carlson 1997; Holstege *et al.* 1998; Chang *et al.* 2001). Moreover, subsequent nutrient deprivation does not appear to result in a significant induction of several of these genes (our unpublished data). Our current model is that these transcription defects result in a failure to undergo a normal growth arrest during nutrient deprivation. As a result, the *rye* mutants fail to assume many of those characteristics normally associated with the stationary phase of growth.

Interestingly, all of the *RYE* genes characterized thus far have been found to encode transcriptional regulators associated with the RNA pol II holoenzyme. The *RYE2*, *RYE3*, and *RYE5* genes were previously shown to encode the Srb11p, Srb9p, and Srb10p proteins, respectively (Chang *et al.* 2001). These proteins are all components of the Srb complex associated with the RNA pol II holoenzyme (Carlson 1997). Like Sin4p, these *SRB* gene products are also required for the proper entry into stationary phase (Chang *et al.* 2001). These results therefore implicate two distinct complexes within the RNA pol II holoenzyme in the control of yeast cell growth: the Srb complex and the Sin4p module of the Mediator. However, the key question that remains is whether the activities of these two complexes are indeed regulated during stationary phase entry. The answer appears to be yes for the Srb complex as the stability of both Srb10p and Srb11p decreases upon nutrient limitation (Cooper *et al.* 1997; Holstege *et al.* 1998). Thus, the Srb complex may be inactivated by the physical removal of particular members of this complex (Holstege *et al.* 1998; Chang *et al.* 2001). This inactivation would result in the increased expression of those genes required during nutrient limitation. However, it is not yet known if either the stability or activity of proteins within the Sin4p module are similarly influenced by nutrient availability.

A novel mode of transcriptional control: The specificity of the genetic interactions observed here suggests that a functional relationship exists between the Ras/PKA signaling pathway and the Sin4p module of the Mediator. One interesting possibility is that Ras/PKA signaling influences RNA pol II activity to ensure that gene expression is properly coordinated with nutrient availability and cell growth. Clearly, the key to understanding this relationship is the identification of the PKA substrate responsible for the above genetic interactions. However, the nature of this target has remained elusive. Our genetics suggests that the relevant PKA substrate is likely not any of the known components of the Sin4p module. This assertion follows from observations that null alleles of *SIN4*, *GAL11*, *HRS1*, and *MED2* are all synthetic lethal with $RAS2^{val19}$; deletion of the relevant target should render the resulting strain insensitive to the effects of elevated Ras/PKA activity. Moreover, none of the proteins in the Sin4p module contain a consensus site for PKA phosphorylation. The best-characterized consensus for the *S. cerevisiae* PKA enzyme fits the format of R-R-*x*-S/T-B, where *x* indicates any amino acid and B indicates a residue with a hydrophobic side-chain (Denis *et al.* 1991). Other potential candidates for this PKA target include proteins encoded by those genes, such as *KIN28*, *SPT20*, and *SRB5*, that have been shown to exhibit a genetic interaction with *sin4* mutations (Valay *et al.* 1995; Roberts and Winston 1997; Chang *et al.* 1999). However, none of these candidates possesses an obvious PKA consensus site either. Thus, we feel that classical genetic approaches may represent the best way to identify this PKA substrate. For this reason, we have initiated a search for extragenic suppressors of the *sin4* $RAS2^{val19}$ synthetic lethality.

The possibility that proteins within the RNA pol II holoenzyme might be direct targets of particular signaling pathways is very intriguing. The proteins within the Srb complex and the Sin4p module of the Mediator appear to control the transcription of distinct subsets of genes (Carlson 1997; Ptashne and Gann 1997; Han *et al.* 1999; Myers and Kornberg 2000). By directly targeting components within these complexes, the cell could bring about rather large changes in gene expression with a single regulatory event. For example, by modulating Sin4p activity, the cell would be able to coordinately control the expression of all promoters affected by this transcriptional regulator. This type of a control mechanism clearly would be more efficient than one where each individual promoter was regulated independently. The ability to effect rather global changes in gene expression would be very useful in those instances where cells undergo significant changes in their overall physiology, such as during the entry into a G_0-like resting state. Although no example of this regulatory mechanism has yet been described, several recent reports have hinted at this type of transcriptional control (Holstege *et al.* 1998; Kuchin *et al.* 2000; Chang *et al.* 2001). The further characterization of the *rye* mutants could therefore provide important insights into the manner in which signaling pathways control gene expression.

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