The C-terminal Domain of the Largest Subunit of RNA Polymerase II Is Required for Stationary Phase Entry and Functionally Interacts with the Ras/PKA Signaling Pathway*

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The Saccharomyces cerevisiae Ras proteins control cell growth by regulating the activity of the cAMP-dependent protein kinase (PKA). In this study, a genetic approach was used to identify cellular processes that were regulated by Ras/PKA signaling activity. Interestingly, we found that mutations affecting the C-terminal domain (CTD) of Rpb1p, the largest subunit of RNA polymerase II, were very sensitive to changes in Ras signaling activity. The Rpb1p CTD is a highly conserved, repetitive structure that is a key site of control during the production of a mature mRNA molecule. We found that mutations compromising the CTD were synthetically lethal with alterations that led to elevated levels of Ras/PKA signaling. Altogether, the data suggested that Ras/PKA activity was negatively regulating a protein that functioned in concert with the CTD during RNA pol II transcription. Consistent with this prediction, we found that elevated levels of Ras signaling caused growth and transcription defects that were very similar to those observed in mutants encoding an Rpb1p with a truncated CTD. In all, these data suggested that S. cerevisiae growth control and RNA pol II transcription might be coupled by using the Ras pathway to regulate CTD function.

The RAS genes encode small GTP-binding proteins that play an important role in regulating cell proliferation in many eukaryotes (1, 2). Ras proteins typically function as molecular switches by oscillating between an active GTP-bound state and an inactive GDP-bound state (3, 4). The budding yeast, Saccharomyces cerevisiae, contains two Ras proteins, Ras1p and Ras2p, that together control the activity of the cAMP-dependent protein kinase, PKA (5, 6). Ras/PKA signaling is a key regulator of growth in this yeast as mutations that inactivate this pathway cause a premature entry into the G0-like resting state, known as stationary phase (4). Conversely, elevated levels of Ras/PKA signaling result in a failure to arrest in a normal stationary phase (4, 6). However, despite the obvious importance of this signaling pathway, only a few PKA substrates important for the control of yeast cell growth have been identified (7–9).

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 (10–12). However, it is not yet known precisely how Ras activity influences the transcriptional apparatus. In S. cerevisiae, RNA polymerase (pol) II exists as a large holoenzyme complex that contains the 12-subunit polymerase, the Mediator co-activator complex, the Srb9-11 protein complex, and several general transcription factors (13). This holoenzyme is recruited to active promoters as a result of specific interactions between Mediator subunits and the DNA-bound transactivators present at these promoters (14, 15). Therefore, there are at least two potential targets for the Ras effects on RNA pol II activity as follows: the various transcription factors bound at the individual promoters, and the regulatory proteins associated with the RNA pol II holoenzyme. A number of studies already indicate that the Ras pathway regulates the activity of specific promoter-bound transcriptional regulators, like Msn2p and Msn4p (9, 16). In contrast, although a few studies (17–19) have hinted at the possibility, there have been no reports of signaling pathways directly targeting components within the RNA pol II holoenzyme.

Although RNA pol II transcription is controlled at multiple levels, it is clear that the C-terminal domain (CTD) of Rpb1p, the largest subunit of RNA pol II, is a key site of regulation. The Rpb1p CTD is a highly conserved, repetitive structure that is required for many aspects of mRNA production including pre-initiation complex formation, transcript elongation, and processing of the nascent transcript (20, 21). In S. cerevisiae, the CTD consists of 26 or 27 repeats of the consensus heptamer, Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (22, 23). The CTD is phosphorylated by multiple protein kinases including Kin28p/Cdk7 in the general transcription factor, TFIIH, Srb10p/Cdk8 in the Srb complex, and Ctk1p/Cdk9 (24). These kinases are generally specific for either serine 2 or serine 5 of the individual repeats. Although all of the details are not yet understood, this phosphorylation clearly plays an important role in the regulation of CTD function (25, 26). For example, the Mediator complex specifically associates with RNA pol II enzymes that contain a hypophosphorylated CTD (27–29). The subsequent phosphorylation of the CTD by Kin28p results in the release of the Mediator and the association of a complex of proteins important for transcriptional elongation (30). Therefore, a complete understanding of the mechanisms regulating mRNA production will require a thorough working knowledge of the Rpb1p CTD.

We are interested in defining the role played by the S. cerevisiae Ras/PKA signaling pathway in the control of stationary phase entry and general cell growth. In this study, we identified specific genetic interactions between mutations af-
fecting the Ras pathway and those affecting the Rpb1p CTD or proteins that function through this domain. These genetic data suggested that the Ras/PAK pathway was negatively regulating activities associated with the Rpb1p CTD. Consistent with this prediction, we found that increased Ras signaling resulted in phenotypes similar to those observed in mutants that encode a Rpb1p with a truncated CTD. These CTD truncation mutants are viable but exhibit specific defects in growth and RNA pol II transcription. Altogether, the data suggest that targets proteins within the RNA pol II holoenzyme is discussed.

EXPERIMENTAL PROCEDURES

Growth Media—Standard Escherichia coli growth conditions and media were used throughout this study (31). Yeast YPAD, 5-fluoroorotic acid, and SC growth media were as described (32, 33). YM-glucose minimal medium containing 0.67% yeast nitrogen base (Difco), 2% glucose, and those growth supplements referred to a yeast minimal medium containing 500 μM methionine. These growth conditions effect repressed expression from the Met3 promoter. The cells were then collected by centrifugation and resuspended in the same growth medium lacking methionine to induce expression from the Met3 promoter.

For the MET3-RAS2V10p experiments, strains carrying this inducible construct were typically grown to mid-log in YM-glucose minimal medium containing 500 μM methionine. These growth conditions effect repressed expression from the Met3 promoter. The cells were then collected by centrifugation and resuspended in the same growth medium lacking methionine to induce expression from the Met3 promoter.

To test for genetic interactions with RAS2V10, the relevant strains were transformed with plasmids containing either RAS2V10p or the inducible MET3-RAS2V10p construct. We tested more than 100 different mutants defective for a wide variety of processes important for cell growth. These strains were either in our lab collection previously or were provided by scientists in the general yeast community. For strains with temperature-sensitive (ts) growth defects, the growth rates of the transformed strains were assessed at temperatures that were permissive or semi-permissive for the growth of the original mutant. In each case, the effects of RAS2V10p on the mutant were compared with the effects on an isogenic wild-type control. Strain information for all of the mutants listed in Table II is available upon request.

Stationary Phase Assays—The stationary phase viability assays were performed as described (12). For each strain tested, the number of survivors after 10–14 days of growth in a minimal medium was normalized to that found for an isogenic wild-type control. The heat shock sensitivity assays were performed on cultures grown for 3 days in a YM-glucose minimal medium at 30 °C. Cells were collected by centrifugation and resuspended in distilled water that was concentrated. The cell suspensions were subjected to a series of 5-fold dilutions, and 200 μl of each suspension was placed into a well of a microtiter plate. These suspensions were then incubated for 3 days at 30 °C, and the relative number of survivors was determined for each strain analyzed.

RNA Analyses—Total RNA was prepared from yeast cells by a hot phenol extraction method described previously (36). For Northern analyses, 20 μg of total RNA per lane was loaded onto a formaldehyde-agarose gel and subjected to electrophoretic separation. The gels were blotted to nylon membranes (Schleicher & Schuell) and probed with the Oligo Labeling Kit (Amersham Biosciences). To measure the amount of total poly(A) RNA, 10–20 μg of total RNA was spotted onto a nitrocellulose membrane with the assistance of a slot blot apparatus (Schleicher & Schuell). The membrane was hybridized with a 32P-labeled oligo(dT) oligonucleotide probe, and the relative signal present was quantified by a Phosphorlmager analysis. As a control for this latter experiment, the amount of poly(A) RNA in an rpb1-1 mutant was measured by Southern blot analysis.
Ras Signaling and the Regulation of RNA pol II Transcription

Table II

| Mutation | Gene product function |
|----------|-----------------------|
| rpb1-104 | Rpb1p CTD truncation  |
| kin28    | TFIIF CTD kinase; Cdk7 |
| ccl1     | Cyclin for Kin28p      |
| tfa1     | Subunit of TFIIE       |
| srb4-138 | Essential subunit of Mediator |
| srb6-107 | Essential subunit of Mediator |
| med6     | Essential subunit of Mediator |
| sin4Δ    | Nonessential subunit of Mediator |
| goll11A  | Nonessential subunit of Mediator |
| rgr1-Δ2  | Essential subunit of Mediator |
| hrs1Δ    | Nonessential subunit of Mediator |
| med2A    | Nonessential subunit of Mediator |

Mutations not exhibiting a synthetic growth defect with RAS2<sup>Val19</sup>

| Mutation | Gene product function |
|----------|-----------------------|
| rpb5-9   | Subunit of RNA pol II |
| sb10A    | CTD kinase; Cdk8      |
| fcp1     | CTD phosphatase       |
| med1A    | Nonessential subunit of Mediator |
| med9A    | Nonessential subunit of Mediator |
| ppr2A    | Pol II transcript elongation factor TFIIF |
| cdc73A   | Subunit of alternative RNA pol II holoenzyme |
| swi2A    | ATPase subunit of SNF/SWI complex |
| cac1A    | Subunit of chromatin assembly factor I |
| gcn5A    | Histone acetylase, subunit of SAGA complex |
| taf9Δ    | TBP-associated protein (TAF) |
| hat1Δ    | Histone acetylase, type B |
| ceg1-237 | Subunit of mRNA capping enzyme |
| prp18    | Splicing factor       |
| prp22    | Required for splicing |
| dbp5     | Required for export of mRNA |
| cdc33-1  | Cap-binding protein; translation factor |
| cdc28-4  | Cdc2 cyclin-dependent kinase homolog |
| cdc37-1  | Required for progression through G<sub>2</sub> |
| sec18-1  | Required for membrane vesicle fusion |
| sec1-1   | Required for transport from the trans-Golgi network to the plasma membrane |
| vps34Δ   | Phosphatidylinositol 3-kinase required for vacuolar protein sorting |
| zdsΔ     | Suppressor of <i>sin4</i> |

was assessed after a shift to the nonpermissive temperature of 37 °C. After 30 min at 37 °C, the poly(A)<sup>+</sup> RNA signal had decreased more than 10-fold in this mutant.

To analyze gene expression at the diauxic shift, log phase cultures were diluted to a density of 0.1 A<sub>600</sub>/ml and were incubated for 8–12 h at 30 °C. The diauxic shift refers to that period of growth where cells have depleted the glucose in the culture and are beginning to utilize the by-products of the fermentation process. For these experiments, total RNA was prepared from cells in late log phase and those undergoing the diauxic shift.

CTD Phosphorylation Assays—Cells were grown to mid-log in minimal medium, collected by centrifugation, and washed with 1 ml of ice-cold 20 mM NaF. Protein extracts for the Rpb1p CTD phosphorylation assays were prepared as described (37). The proteins were separated on a 5% SDS-polyacrylamide gel and were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) (36). The membrane was hybridized with antisera specific for the nonphosphorylated CTD repeat, monoclonal SWG16 (Covance Research Products); phosphoserine at position 2 of the CTD repeats, monoclonal H5 (kindly provided by David Bregman); and phosphoryserine at position 5, monoclonal H14 (Covance Research Products). Immunoreactive proteins were detected with anti-mouse IgG (Amersham Biosciences) used at a dilution of 1:2000. The Supersignal chemiluminescent substrate (Pierce) was subsequently used to illuminate the reactive bands.

RESULTS

Elevated Levels of Ras/PKA Signaling Were Synthetically Lethal With Mutations That Affect RNA pol II Transcription—We are interested in understanding the role played by the Ras/ PKA signaling pathway in the control of growth in <i>S. cerevisiae</i>. To this end, we have been examining the effects of Ras signaling activity on mutants defective in a variety of cellular processes. In particular, these experiments have been directed at identifying mutant phenotypes that were either suppressed or enhanced by increased levels of Ras signaling.

The underlying rationale was that processes controlled by the Ras/PKA pathway would be the most sensitive to changes in Ras signaling activity. Interestingly, we found that many mutants defective for RNA pol II transcription exhibited a severe growth defect in the presence of increased Ras/PKA activity (Fig. 1A and Table II). These Ras effects were rather specific for RNA pol II transcription as mutations affecting other cellular processes were relatively insensitive to increased Ras activity (Table II) (38). These latter mutations included those affecting protein translation, cell cycle progression, protein secretion, and mRNA export and processing.

These genetic studies have converged upon an ongoing analysis in our laboratory of the yeast rye mutants. These mutants were originally identified on the basis of a defective transcriptional response to nutrient deprivation (12). A subsequent analysis has shown that the rye mutants are defective for the control of growth and are unable to enter into a normal stationary phase (12, 19). During the course of these studies, we found that rye1 mutations were synthetically lethal with a hyperactive allele of <i>RAS2</i>, known as <i>RAS2<sup>Val19</sup></i> (19). This allele encodes a Ras2p with diminished GTPase activity, and thus the altered Ras2p is found more often in the active GTP-bound form (5). The <i>RYE1</i> gene was cloned and found to be identical to <i>SIN4</i>, a gene that encodes a component of the yeast Mediator complex (19, 39). Thus, two independent lines of investigation have identified a genetic interaction between the Ras/PKA signaling pathway and the RNA pol II transcription apparatus.

In all, more than 100 different mutations were examined in combination with <i>RAS2<sup>Val19</sup></i> and other alterations that affect the Ras/PKA signaling pathway (Table II and data not shown). Several examples of the synthetic growth defects observed with <i>RAS2<sup>Val19</sup></i> are shown in Fig. 1. The presence of increased Ras signaling greatly exacerbated the growth defects associated with <i>sin4</i>, <i>kin28</i>, <i>srb4-138</i>, and rpb1-104 mutants. <i>SRB4</i> encodes a component of the Mediator, and the <i>rpb1-104</i> allele encodes a truncated Rpb1p protein that contains only 11 of the 26 CTD repeat units (40, 41). These experiments employed an inducible allele of <i>RAS2<sup>Val19</sup></i> in which the RAS2 promoter was replaced with the promoter from the yeast <i>MET3</i> gene (19). The <i>MET3</i> promoter is repressed by the presence of methionine in the growth medium, and <i>RAS2<sup>Val19</sup></i> is therefore expressed only when methionine is absent (42, 43). The growth defects observed with the <i>MET3-RAS2<sup>Val19</sup></i> allele in Fig. 1 were specific to media lacking methionine (data not shown). Finally, all pairwise double mutant combinations between the <i>RAS2<sup>Val19</sup></i>, <i>kin28</i>, <i>rpb1-104</i>, and <i>sin4</i> mutations resulted in a severe growth defect (Fig. 1B) (44). This result indicated that each of these mutations was very sensitive to a further decrease in transcriptional efficiency and suggested that Ras/PKA signaling might also be influencing RNA pol II activity.

In addition to the above growth defects, the presence of increased levels of Ras/PKA signaling resulted in a rapid cell death in mutants that were defective for RNA pol II transcription. For example, with <i>sin4</i> mutants, less than 0.01% of the cells were viable 2 h after induction of <i>RAS2<sup>Val19</sup></i> expression (Fig. 1C). A similar induction of <i>RAS2<sup>Val19</sup></i> in wild-type cells did not affect cell growth or viability. Because <i>Sin4p</i> is a transcriptional regulator, this lethality could have been due to a global defect in RNA pol II transcription or to defects at a subset of genes essential for cell viability. Two experimental observa-
mutations indicated that this latter possibility was a more likely explanation. First, the expression of RasSignaling resulted in a rapid cell death in mutants defective for RNA pol II transcription. A, mutations that compromise RNA pol II function exhibited a synthetic growth defect with RasSignaling. Yeast strains with the indicated mutations were transformed with either the control vector, pRS416 (RAS2), or a plasmid with RasSignaling under the control of the inducible promoter from the yeast MET3 gene, pPHY795 (RAS2Val19). The strains were grown to mid-log in the presence of methionine (repressed), and equal numbers of cells were then spotted to a growth medium lacking methionine (induced). Growth was assessed after 2 days at 30 °C for wild-type, sin4, kin28, and srb4-138 and at 33 °C for rpb5-9 and rpb1-104. The strains shown are wild-type (PHY1220), sin4 (PHY1454), kin28 (JGV4), srb4-138 (Z551), rpb5-9 (WY-186), and rpb1-104 (Z551). For all strains, the observed growth rate was compared with that of an isogenic wild-type control. B, schematic showing the synthetic lethal interactions observed between the indicated mutations. The lines with barbed ends indicate the synthetic lethal interactions detected in this study. The straight line indicates an interaction described previously (44). C, increased Ras signaling caused a rapid cell death in sin4 mutants. Yeast strains were grown to mid-log in a minimal medium containing methionine and were transferred to a medium lacking methionine to induce expression from MET3-RasSignaling. At the indicated times, dilutions were plated to a rich growth medium and incubated for 3 days at 30 °C. The number of colonies formed was a measure of the number of viable cells present in the culture. In each case, the numbers were normalized to those obtained for the isogenic wild-type strain. The strains analyzed were as follows: wild-type (PHY1837, triangles), RasSignaling (PHY1834, circles), sin4 (PHY1647, diamonds), and sin4 RasSignaling (PHY1649, squares). The RasSignaling strains contained the MET3-RasSignaling allele. D, increased Ras/PKA signaling did not cause a global defect in mRNA accumulation in sin4 mutants. Total poly(A)+ RNA levels in wild-type and sin4 RasSignaling cells were measured by a slot blot analysis. Wild-type (PHY1837) and sin4 MET3-RasSignaling (PHY1649) strains were grown to mid-log at 30 °C in a YM-glucose minimal medium containing 500 μM methionine. The cells were then transferred to a minimal medium lacking methionine to induce expression from the MET3-RasSignaling gene. Total RNA was prepared at 0 and 4 h after the transfer to the medium lacking methionine, and 20 μg of each sample was spotted onto a nitrocellulose membrane. The level of poly(A)+ RNA was then assessed by hybridization with a 32P-labeled oligo(T) probe. The relative signal was quantified with a PhosphorImager and is shown in terms of arbitrary PhosphorImager units.

interpret the lethality of the double mutants to be due to the misregulation of a limited number of promoters that have a specific function in growth control. The RasSignaling Growth Defects Were Specific to Mutations Affecting the Rpb1p CTD—It is important to note that not all mutations affecting RNA pol II transcription were influenced by the presence of RasSignaling (Table II). Instead, most of the mutations that were synthetically lethal with RasSignaling affected proteins that interacted with the Rpb1p CTD. For example, RasSignaling was synthetically lethal with mutations that affected the Mediator (sin4, gal11, med6, and srb4), the TFIIH complex (kin28, ccl1, rig2, and tfa1) and the CTD itself (Table II) (45, 46). The Mediator associates with the RNA pol II holoenzyme by directly interacting with the CTD, whereas TFIIH contains a major CTD kinase. Kin28p (28, 47, 48). The importance of the CTD for these RasSignaling effects was perhaps best illustrated by the responses of different rpb1 alleles to increased Ras signaling. The presence of RasSignaling greatly exacerbated the growth defects associated with those rpb1 alleles that encoded a truncated CTD (Fig. 2A). In contrast, rpb1 mutations that affected other regions of Rpb1p were relatively insensitive to increased Ras activity (Fig. 2A). These latter mutations typically cause a severe ts defect in RNA pol II

FIG. 1. Increased Ras signaling resulted in a rapid cell death in mutants defective for RNA pol II transcription. A, mutations that compromise RNA pol II function exhibited a synthetic growth defect with RasSignaling. Yeast strains with the indicated mutations were transformed with either the control vector, pRS416 (RAS2), or a plasmid with RasSignaling under the control of the inducible promoter from the yeast MET3 gene, pPHY795 (RAS2Val19). The strains were grown to mid-log in the presence of methionine (repressed), and equal numbers of cells were then spotted to a growth medium lacking methionine (induced). Growth was assessed after 2 days at 30 °C for wild-type, sin4, kin28, and srb4-138 and at 33 °C for rpb5-9 and rpb1-104. The strains shown are wild-type (PHY1220), sin4 (PHY1454), kin28 (JGV4), srb4-138 (Z551), rpb5-9 (WY-186), and rpb1-104 (Z551). For all strains, the observed growth rate was compared with that of an isogenic wild-type control. B, schematic showing the synthetic lethal interactions observed between the indicated mutations. The lines with barbed ends indicate the synthetic lethal interactions detected in this study. The straight line indicates an interaction described previously (44). C, increased Ras signaling caused a rapid cell death in sin4 mutants. Yeast strains were grown to mid-log in a minimal medium containing methionine and were transferred to a medium lacking methionine to induce expression from MET3-RasSignaling. At the indicated times, dilutions were plated to a rich growth medium and incubated for 3 days at 30 °C. The number of colonies formed was a measure of the number of viable cells present in the culture. In each case, the numbers were normalized to those obtained for the isogenic wild-type strain. The strains analyzed were as follows: wild-type (PHY1837, triangles), RasSignaling (PHY1834, circles), sin4 (PHY1647, diamonds), and sin4 RasSignaling (PHY1649, squares). The RasSignaling strains contained the MET3-RasSignaling allele. D, increased Ras/PKA signaling did not cause a global defect in mRNA accumulation in sin4 mutants. Total poly(A)+ RNA levels in wild-type and sin4 RasSignaling cells were measured by a slot blot analysis. Wild-type (PHY1837) and sin4 MET3-RasSignaling (PHY1649) strains were grown to mid-log at 30 °C in a YM-glucose minimal medium containing 500 μM methionine. The cells were then transferred to a minimal medium lacking methionine to induce expression from the MET3-RasSignaling gene. Total RNA was prepared at 0 and 4 h after the transfer to the medium lacking methionine, and 20 μg of each sample was spotted onto a nitrocellulose membrane. The level of poly(A)+ RNA was then assessed by hybridization with a 32P-labeled oligo(T) probe. The relative signal was quantified with a PhosphorImager and is shown in terms of arbitrary PhosphorImager units.

FIG. 2. Evidence for a functional interaction between the Ras/PKA signaling pathway and the RNA pol II CTD. A, rpb1 alleles that encode CTD truncations were sensitive to increased levels of Ras/PKA signaling. The indicated rpb1 strains with either a control vector (RAS2) or a plasmid containing MET3-RasSignaling (RAS2Val19) were grown to mid-log in the presence of methionine. Equal numbers of cells were then spotted to a growth medium lacking methionine to induce expression from the MET3 promoter and grown for 3 days at 33 °C. The scheme indicates the relative positions of the mutations in the rpb1 alleles. B, decreased signaling through the Ras/PKA pathway suppressed the growth defects associated with kin28 and tfa1 mutants. Wild-type (GF262-2), kin28 (JGV4), and tfa1 (YSB0331) strains containing either a control vector or a high copy PDE2 plasmid were grown for 3 days on a YM-glucose minimal medium at 32 °C for kin28 and 35 °C for tfa1.

The RasSignaling Growth Defects Were Specific to Mutations Affecting the Rpb1p CTD—It is important to note that not all mutations affecting RNA pol II transcription were influenced by the presence of RasSignaling (Table II). Instead, most of the mutations that were synthetically lethal with RasSignaling affected proteins that interacted with the Rpb1p CTD. For example, RasSignaling was synthetically lethal with mutations that affected the Mediator (sin4, gal11, med6, and srb4), the TFIIH complex (kin28, ccl1, rig2, and tfa1) and the CTD itself (Table II) (45, 46). The Mediator associates with the RNA pol II holoenzyme by directly interacting with the CTD, whereas TFIIH contains a major CTD kinase. Kin28p (28, 47, 48). The importance of the CTD for these RasSignaling effects was perhaps best illustrated by the responses of different rpb1 alleles to increased Ras signaling. The presence of RasSignaling greatly exacerbated the growth defects associated with those rpb1 alleles that encoded a truncated CTD (Fig. 2A). In contrast, rpb1 mutations that affected other regions of Rpb1p were relatively insensitive to increased Ras activity (Fig. 2A). These latter mutations typically cause a severe ts defect in RNA pol II.
activity and have been described previously (49). These data therefore suggested the existence of a functional relationship between Ras signaling activity and the Rpb1p CTD.

Because the CTD is essential for multiple steps during the production of the mature mRNA, we examined mutations that affected each of these different steps. This analysis indicated that the RAS2Val19 lethality was limited to those mutations affecting RNA pol II transcription. Mutations that affected later steps, such as mRNA splicing and export, did not exhibit a genetic interaction with RAS2Val19 (Table II). In addition, the Ras effects were specific to particular components of the RNA pol II machinery. Mutants defective for other activities, including other subunits of RNA pol II (rpb5), a CTD-specific phosphatase (fcp1), and a second CTD kinase (srb10), were not affected by increased levels of Ras/PKA signaling (Table II). In all, the specificity of the genetic interactions suggested that the Ras/PKA pathway might be targeting the CTD itself or perhaps a protein that functionally interacts with the CTD during RNA pol II transcription. Moreover, the specificity of the RAS2Val19 effects was consistent with the possibility that a single PKA target was responsible for the genetic interactions observed in this study.

**Decreased Ras/PKA Signaling Suppresses Mutations That Affect RNA pol II Transcription**—The presence of RAS2Val19 results in relatively high levels of PKA activity, levels higher than those typically seen during normal yeast cell growth (4, 6). Therefore, we tested whether variations within the normal physiological spectrum of Ras activity would also have an effect upon RNA pol II transcription. Indeed, the above data suggest that decreasing the levels of Ras signaling might suppress mutations affecting CTD-related functions. Consistent with this prediction, we found that the ts growth defects associated with kin28 and tfa1 mutants were suppressed by lowering the activity of the Ras/PKA signaling pathway (Fig. 2B). TFA1 encodes the large subunit of TFIIE, and as indicated above, KIN28 encodes a CTD kinase associated with TFIIH (48, 50). The general transcription factor, TFIIH, is required for the recruitment of TFIIH to the Rpb1p CTD (51–53). In these experiments, Ras/PKA activity was lowered by the introduction of a high copy plasmid containing the PDE2 gene. PDE2 encodes a cAMP phosphodiesterase that, when overproduced, results in lowered levels of cAMP and in decreased PKA activity (54). This PDE2 plasmid had no significant effect on the growth of wild-type cells but allowed kin28 and tfa1 ts strains to grow at elevated temperatures (Fig. 2B). In addition, a recent study (19) found that cyr1 mutations that lower cAMP levels also suppress transcription defects associated with sin4 mutants. CYR1 encodes the adenyl cyclase that is activated by the yeast Ras proteins (55). Therefore, both raising and lowering the mitotic levels of Ras signaling had profound effects upon mutations that affect Rpb1p CTD function.

**RAS2Val19 Mutants Exhibited Growth and Transcription Defects Similar to Those Associated with CTD Truncation Mutants**—The above data can be explained by a model proposing that Ras activity negatively regulates aspects of Rpb1p CTD function. Such a model would predict that elevated levels of Ras signaling would produce phenotypes similar to those associated with rpb1 alleles that compromise CTD function. For these experiments, we used the rpb1-104 allele that encodes an Rpb1p with only 11 CTD repeats; the presence of at least 8–10 heptad repeats is required for cell viability (56, 57). Previous work has shown that rpb1-104 mutants grow poorly on media lacking inositol (49), and we have found that these mutants exhibit growth defects on galactose media (Fig. 3A). Here we found that RAS2Val19 mutants exhibited similar defects on these growth media (Fig. 3A). In addition, these growth defects were exacerbated in RAS2Val19 rpb1-104 double mutants (Fig. 3A). Note that this double mutant was viable at 30 °C on glucose-based media but exhibited a severe growth defect on such media at both higher and lower temperatures (data not shown).

The RAS2Val19 and rpb1-104 mutants also exhibited similar defects in activated transcription. CTD truncation mutants had been shown previously to be defective for transcription from the INO1 and GAL1 promoters (58). For both genes, the expression under inducing conditions was down 5–8-fold in the rpb1-104 mutant (Fig. 3B). A similar transcription defect was observed with RAS2Val19 mutants, and these defects were greatly exacerbated in the RAS2Val19 rpb1-104 double mutant (Fig. 3B). Moreover, RAS2Val19 had no effect on the induced levels of other promoters, like those for PHO5 and MET19, that were also insensitive to CTD truncations (Fig. 3B and data not shown). Therefore, increased levels of Ras/PKA signaling re-
sulted in growth and transcription phenotypes similar to those previously associated with CTD truncations.

The Rpb1p CTD Was Required for Entry into a Normal Stationary Phase upon Nutrient Deprivation—We also tested whether rpb1 alleles that truncate the CTD caused growth defects that were normally associated with increased levels of Ras signaling. This would be expected if some of the growth consequences of elevated Ras activity were due to the down-regulation of CTD function. The primary defect associated with yeast RAS2Val19 mutants is a failure to arrest within stationary phase when an essential nutrient becomes limiting (4). This defect in stationary phase entry has a number of consequences including a rapid loss of viability during growth in a nutrient-limiting medium, a failure to accumulate storage carbohydrates, like glycogen, and a diminished resistance to environmental stresses, including heat shock (4, 6, 10, 59). We tested whether rpb1-104 mutants were defective for stationary phase entry by examining each of these characteristics.

Wild-type stationary phase cells can survive for extended periods in a nutrient-depleted growth medium. In contrast, RAS2Val19 mutants exhibit a rapid loss of viability following nutrient deprivation (4, 6). For example, after 10 days of growth in minimal medium, the number of survivors in a RAS2Val19 culture was less than 1% that observed with an isogenic wild-type strain (Fig. 4A). We found that rpb1-104, sin4, and kin28 mutants all exhibited a similar stationary phase viability defect (Fig. 4A). In each case, the survival rate of the mutant after 14 days of growth was more than 15-fold lower than that of the wild-type control (Fig. 4A). Thus, the presence of a wild-type Rpb1p CTD was required for the normal acquisition of this stationary phase characteristic.

Stationary phase cells also accumulate elevated levels of the storage carbohydrate, glycogen, and exhibit an increased resistance to elevated temperatures (10). We found that rpb1-104 mutants were defective for both of these stationary phase traits. First, rpb1-104 mutants, like RAS2Val19, failed to accumulate normal levels of glycogen following nutrient deprivation (Fig. 4B). For these assays, glycogen levels were assessed in wild-type, RAS2Val19, and rpb1-104 cultures grown for 2, 4, or 6 days in minimal medium at 30 °C. The rpb1-104 storage defect was already apparent after only 2 days of growth in minimal medium, a time that roughly corresponds to the beginning of the post-diauxic phase of growth (12). Finally, we found that rpb1-104 mutants grown for 3 days in minimal medium were 5–6 times more sensitive than wild-type cells to a brief heat shock (Fig. 4C). In all, these data indicated that the Rpb1p CTD was required for entry into a normal stationary phase in S. cerevisiae. Moreover, these results were consistent with the proposition that Ras/PKA signaling negatively regulates functions associated with the Rpb1p CTD.

It is important to point out that the stationary phase defects associated with rpb1-104 mutants were generally less severe than those observed with RAS2Val19. For example, although RAS2Val19 mutants exhibited a dramatic loss of viability after 5 days of growth in minimal medium, similar defects in the rpb1-104 strains were not observed until 10–14 days of growth. One possible explanation for this difference is that the Ras/PKA pathway targets multiple proteins important for stationary phase entry. Components associated with the Rpb1p CTD would represent only one of these regulatory targets. Alternatively, the less severe phenotype of the rpb1-104 mutant could be due to the presence of the 11 CTD repeats in the encoded Rpb1p; an Rpb1p lacking all repeats might be severely defective in stationary phase entry. Unfortunately, because the Rpb1p CTD is essential for cell viability, this latter possibility would be difficult to test directly.

RAS2Val19 and rpb1-104 Mutants Exhibited Similar Defects in RNA pol II Transcription upon Nutrient Deprivation—The entry into stationary phase is accompanied by broad changes in the patterns of gene expression. Although the levels of most transcripts decrease upon nutrient deprivation, specific sets of genes are induced at this time (10, 11, 60). Many of these latter genes are negatively regulated by Ras/PKA signaling activity (60, 61). In this section, we tested whether the Rpb1p CTD was required for the full induction of seven genes known to be
regulated by nutrient limitation and the Ras/PKA signaling pathway. The expression of four of these genes, CTT1, GUT2, PGM2, and YOR173w, is dependent upon the Msn2p and Msn4p transcriptional regulators (60, 62, 63). Msn2p and Msn4p are key mediators of the yeast cell response to environmental stress and are themselves regulated by Ras/PKA activity (9, 64). The other three genes, APE2, UGA1, and UGA2, are regulated by Ras signaling activity in a Msn2p/Msn4p-independent manner (60). These genes are all expressed at a low level during mitotic growth and are induced during the diauxic shift (data not shown) (11, 60, 61).

For this study, we examined the expression levels of these Ras-regulated genes at times that correlated roughly with late log phase and the diauxic shift (Fig. 5A). The levels present in the RAS2Val19 and rpb1-104 mutants were compared with those in the isogenic wild-type strain. As expected, we found that all seven genes were induced at the diauxic shift in wild-type cells and that this induction was impaired in the RAS2Val19 mutant (Fig. 5B). In general, the expression levels were 2–10-fold lower in the RAS2Val19 strain (Fig. 5C). Interestingly, the induced level of all of these genes, except GUT2, was found to be diminished to a similar extent in the rpb1-104 mutant (Fig. 5, B and C). These data indicated that rpb1-104 mutants were defective in the initial transcriptional response to nutrient deprivation. The subsequent stationary phase phenotypes observed with these mutants are likely a consequence of these transcription defects.

**Elevated Levels of Ras/PKA Signaling Did Not Affect the Phosphorylation State of the Rpb1p CTD**—Because many of the functions of the Rpb1p CTD are regulated by serine phosphorylation (21, 24), we examined the phosphorylation state of this domain in RAS2Val19 mutants. Previous work (65) has shown that Rpb1p is found in two forms in vivo: a highly phosphorylated form, known as IIa, and a hypophosphorylated form, known as IIb. The degree of CTD phosphorylation is indicated by the relative amount of Rpb1p found in the IIb form. We found that RAS2Val19 and wild-type cells contained a similar fraction of Rpb1p in the highly phosphorylated IIa form (Fig. 6A).

The phosphorylation state of the Rpb1p CTD was also assessed by immunoblotting experiments with antibodies that specifically recognize the phosphorylated forms of the serines at positions 2 and 5 of the CTD repeats (37, 66). The relative signal in the immunoblot serves as a measure of the extent of phosphorylation at that particular serine residue. This analysis also indicated that RAS2Val19 had no significant effect on the phosphorylation state of the Rpb1p CTD. A, analysis of CTD phosphorylation in RAS2Val19 mutants. The relative level of Rpb1p phosphorylation was assessed by immunoblot analysis with antibodies that recognize unphosphorylated CTD repeats (α-CTD), monomonal 8WG16) and phosphorylated serine 2 (α-Ser-P2; monomonal H5) or serine 5 (α-Ser-P5; monomonal H14) residues in the CTD repeats. Protein extracts were prepared from mid-log cultures of wild-type or RAS2Val19 cells as described under “Experimental Procedures.” Relative protein concentrations were assessed by Bradford assays and immunoblotting with antibodies to phosphoglycerate kinase (PGK) and Vp34p, Dpb5p, and carboxypeptidase Y (data not shown). The strains analyzed were PHY1220 containing either a control vector, pRS416 (RAS2), or a RAS2Val19 plasmid, pJR1040 (RAS2Val19). B, CTD phosphorylation levels decreased in kin28 mutants. Top, CTD phosphorylation was assessed by Western immunoblotting with the 8WG16 monoclonal antibody as described in A. Protein extracts were prepared from wild-type (WT), RAS2Val19, and rpb1-104 (JGV4) cells after a 0- and 30-min shift to the nonpermissive temperature of 39 °C. Bottom, the levels of serine 5 phosphorylation in the CTD repeats were assessed by Western immunoblotting with monoclonal antibody H-14. Following a 30-min incubation at 39 °C, protein extracts were prepared from wild-type (GF262-2, RA), RAS2Val19 (GF262-2 with pJR1040, RAS2Val19), and kin28 (JGV4; kin28) cells. The levels of phosphoglycerate kinase served as a loading control for this experiment.
the relative amount of phosphorylation at either serine 2 or serine 5 (Fig. 6A). As a control for antibody specificity, CTD phosphorylation levels were also assessed in a kin28 Δ mutant. KIN28 encodes a CTD kinase, and the inactivation of Kin28p results in a significant decrease in the level of CTD phosphorylation (44, 67). Following a 30-min incubation at the nonpermissive temperature, we found that the CTD phosphorylation signal detected with the SWG16, H5, and H14 antibodies was reduced to background levels in the kin28 Δ strain (Fig. 6B and data not shown). Therefore, the effects of elevated Ras signaling on RNA pol II transcription did not involve a gross alteration in the pattern of CTD phosphorylation.

The sin4 RAS2Val19 Synthetic Lethality Was Suppressed by Mutations Affecting the Mediator—Previous studies have identified mutations that suppress the growth defects associated with truncations of the Rpb1p CTD. These SRB mutations were subsequently found to reside in genes encoding components of the Mediator (SRB2 and SRB4-7) and Srb (SRB8-11) complexes found in the RNA pol II holoenzyme (40, 41, 68). Both of these complexes physically interact with the Rpb1p CTD and influence RNA pol II transcription (24, 69). We therefore tested whether these SRB mutations would also suppress the RAS2Val19 rpb1-104 synthetic lethality. Interestingly, only those SRB mutations that affected Mediator components were able to suppress the Ras-induced lethality (Fig. 7A). These results indicated that the two classes of SRB mutation were functionally distinct and that simply suppressing the growth defects associated with CTD truncations might not be sufficient to alleviate the lethal effects of RAS2Val19. In addition, these data ruled out models proposing that the Ras effects on RNA pol II transcription observed here were due to the Ras/PKA stimulation of the inhibitory activity associated with the Srb complex. The Srb complex has been shown to function as a negative regulator of transcription at a select set of promoters (24).

Finally, to gain some insight into the nature of the Ras effects on CTD function, we tested whether the dominant SRB mutations would also suppress the growth defects associated with RAS2Val19. This suppression might be expected if Ras activity was directly affecting the Rpb1p CTD. However, we found that neither SRB4-1, SRB5-1, nor SRB6-1 was able to suppress the Ino- or Gal- growth defects of RAS2Val19 mutants (data not shown). In addition, neither of these SRB alleles suppressed the growth defects associated with kin28 and tfa1 mutations (data not shown). In contrast, these kin28 and tfa1 defects were suppressed by decreasing the level of Ras/PKA signaling in these mutants (see above). Interestingly, the Srb2p, Srb4p, Srb5p, and Srb6p proteins appear to form a core subcomplex within the Mediator that is in close contact with the RNA pol II enzyme (70, 71). This core complex may be responsible for relaying signals received from other Mediator proteins to the polymerase; these other Mediator proteins would be the components that physically interact with DNA-bound transactivators. With such an architecture, our genetic data would suggest that the Ras/PKA pathway is targeting a component that functions either downstream of the Mediator or in an independent pathway that works through the Rpb1p CTD to regulate RNA pol II transcription.

DISCUSSION

This report describes a functional interaction that exists between the S. cerevisiae Ras/PKA signaling pathway and the C-terminal domain, or CTD, of Rpb1p, the largest subunit of RNA pol II. This connection was initially suggested by the lethality observed when Ras/PKA activity was elevated in mutants defective for RNA pol II transcription. The ensuing analysis indicated that the Rpb1p CTD was critical for these genetic interactions as mutations that compromised CTD function were the most sensitive to changes in Ras/PKA signaling activity. Altogether, the data suggested that Ras/PKA activity was influencing RNA pol II transcription at select promoters by regulating the function of the Rpb1p CTD. Because Ras/PKA signaling did not significantly affect the phosphorylation state of the CTD, we suggest that the Ras/PKA pathway is instead targeting proteins that function in concert with the Rpb1p CTD.

In the simplest sense, two types of models could be invoked to explain the genetic data presented here. In the first, the Ras/PKA pathway would be targeting some process unrelated to RNA pol II transcription. The observed growth defects would then be due to the additive effects of compromising this second process in a cell that is already defective for RNA pol II transcription. In the other model, the Ras/PKA pathway would be directly targeting a component of the RNA pol II transcription apparatus. In this case, the growth defects would be due to the synergistic effects of inhibiting transcription at two possibly related points. We feel that the data presented here are better explained by the latter model for the following reasons. First, the genetic interactions observed with the Ras/PKA pathway...
were limited to mutations affecting particular aspects of RNA pol II transcription; not all mutations affecting RNA pol II transcription were influenced by Ras signaling activity. In fact, many of the mutations that did not exhibit a synthetic lethal interaction with RAS2Val19 result in a much more severe defect in transcription than do the Rpb1p CTD truncations. Second, increasing the levels of Ras/PKA signaling resulted in phenotypes reminiscent of those associated with a loss of CTD function and vice versa. Third, decreased signaling through the Ras/PKA pathway suppressed the growth defects associated with kin28 and tfa1 mutants. Both Kin28p and Tfa1p affect RNA pol II transcription, in part, by regulating the phosphorylation of the Rpb1p CTD (46). Fourth, not all of the srb mutations suppressed the RAS2Val19 rpb1-104 synthetic lethality. All of these srb mutations suppress the growth defects associated with CTD truncations, and the first model would predict that these srb mutations would suppress the double mutant lethality as well. Altogether, these data suggest that the RAS2Val19 lethality observed here is not merely the simple sum of a defect in transcription coupled with a defect in some other unrelated process important for cell growth. Instead, the data appear to support a model where Ras/PKA activity targets proteins important for RNA pol II transcription.

Clearly, one of the main priorities for future work will be the identification of the PKA substrate responsible for the genetic effects characterized in this study. Previous work with the yeast and mammalian PKA enzymes has identified a consensus target site of RRXS/T/B, where X indicates any amino acid and B refers to those amino acids with a hydrophobic side chain (72, 73). Unfortunately, there are few candidate proteins within the RNA pol II transcription apparatus that contain a perfect match to this consensus. One of the best potential sites we identified was an RRRSS sequence present within the Mediator protein, Med1p (74). However, our analysis of Med1p has indicated that this protein is not the PKA target responsible for the genetic interactions observed in this study. This inability to identify the PKA substrate by sequence comparison is not altogether surprising. One of the primary difficulties encountered during the study of protein kinases is the variation typically found in the target sites that are phosphorylated (75). Therefore, the relevant PKA target could possess a phosphorylation site that varies enough from the consensus that it would be rather difficult to recognize a priori. As a result, we are presently carrying out more classical genetic approaches that do not require any prior knowledge of the nature of the PKA target. One example of such a strategy is a genetic screen that aims to identify mutations that suppress the synthetic lethality of the rpb1-104 RAS2Val19 double mutant. Such genetic approaches should allow us to identify the PKA substrate responsible for the Ras effects on RNA pol II transcription that are described in this study.

In all, the data presented here suggest that the Ras/PKA pathway is negatively regulating proteins that function through the Rpb1p CTD. At first glance, this might appear to be counterintuitive as both Ras/PKA activity and the CTD are essential for yeast cell growth. However, a possible explanation is suggested by studies of rpb1 alleles that encode proteins with a truncated CTD. These rpb1 mutants, like rpb1-104, exhibit defects in expression from a distinct set of genes; the transcription from many, and perhaps most, promoters is apparently unaffected by the shortening of the CTD (58, 76). This result indicates that particular genes require more CTD activity for their normal levels of expression. Therefore, the Ras pathway could affect the expression of specific sets of genes by down-regulating the activities of proteins associated with the Rpb1p CTD. Because Ras signaling is generally thought to serve as an indicator of environmental growth conditions (4, 9, 38), the affected genes should include those that would be preferentially expressed under conditions that are less than ideal for yeast cell growth. Clearly, this correlation holds for most of the genes examined in this study. In wild-type cells, the effects of the Ras pathway would presumably not be able to significantly dampen the expression of those genes required for growth. However, compromising the RNA pol II machinery by a second mutation could cause RAS2Val19 to limit expression from essential genes and thus result in the synthetic lethality observed in this study.

The possibility that the Ras/PKA pathway could be regulating gene expression by targeting components within the RNA pol II holoenzyme is especially intriguing. Although no definitive examples have yet been described, the possibility of this type of a control mechanism has been alluded to in a number of previous studies (12, 17, 18, 77–79). In essence, this model suggests that in addition to targeting DNA-bound transcriptional regulators, signaling pathways might also regulate the activities of components within the RNA pol II holoenzyme (Fig. 7B). In this manner, signal transduction pathways would be able to control transcription from a large number of promoters in a single regulatory step. This type of an approach would be an efficient and parsimonious way to bring about coordinated changes in the patterns of gene expression. Indeed, this type of a mechanism might be used by the Snf1p protein kinase. Snf1p has been shown to associate physically with the RNA pol II holoenzyme and thereby elicit a significant change in the yeast transcriptosome (17). In addition, the holoenzyme proteins, Srb9p, Srb10p, and Srb11p, are likely targets of a signal transduction pathway responsible for coordinating yeast cell growth with nutrient availability (12, 77). Finally, the data presented in this report suggest that the Ras/PKA signaling pathway influences RNA pol II transcription by modulating activities associated with the Rpb1p CTD. Further studies will be necessary to ascertain whether this type of a regulatory mechanism is indeed used to control gene expression in eukaryotic cells.

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The C-terminal Domain of the Largest Subunit of RNA Polymerase II Is Required for Stationary Phase Entry and Functionally Interacts with the Ras/PKA Signaling Pathway

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