The Ras/PKA Signaling Pathway May Control RNA Polymerase II Elongation via the Spt4p/Spt5p Complex in *Saccharomyces cerevisiae*

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ABSTRACT

The Ras signaling pathway in *Saccharomyces cerevisiae* controls cell growth via the cAMP-dependent protein kinase, PKA. Recent work has indicated that these effects on growth are due, in part, to the regulation of activities associated with the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. However, the precise target of these Ras effects has remained unknown. This study suggests that Ras/PKA activity regulates the elongation step of the RNA polymerase II transcription process. Several lines of evidence indicate that Spt5p in the Spt4p/Spt5p elongation factor is the likely target of this control. First, the growth of *spt4* and *spt5* mutants was found to be very sensitive to changes in Ras/PKA signaling activity. Second, mutants with elevated levels of Ras activity shared a number of specific phenotypes with *spt5* mutants and vice versa. Finally, Spt5p was efficiently phosphorylated by PKA *in vitro*. Altogether, the data suggest that the Ras/PKA pathway might be directly targeting a component of the elongating polymerase complex and that this regulation is important for the normal control of yeast cell growth. These data point out the interesting possibility that signal transduction pathways might directly influence the elongation step of RNA polymerase II transcription.

THE control of RNA polymerase (pol) II transcription occurs at multiple levels including promoter recognition, mRNA chain initiation, promoter escape, transcript elongation, and mRNA chain termination (Lee and Young 2000). Although each of these steps is critical for the ultimate production of a mature mRNA molecule, most early studies of transcription focused on the regulation of the initial event of promoter recognition. However, work from the past several years has begun to unravel the mechanisms regulating RNA pol II transcript elongation (Uptain *et al.* 1997; Conaway *et al.* 2000; Hartzog *et al.* 2002). This work has been spurred on, at least in part, by observations linking several protein factors important for elongation to a variety of human diseases and to the propagation of the human immunodeficiency virus (Braddock *et al.* 1991; Marciniak and Sharp 1991; Krumm and Groudine 1995; Wada *et al.* 1998a; Conaway and Conaway 1999). Altogether, these studies have suggested that the transcription rate of a significant fraction of eukaryotic genes is regulated at the level of elongation.

A series of biochemical and genetic studies have identified a number of protein factors that control RNA pol II transcript elongation (Conaway *et al.* 2000; Hartzog *et al.* 2002). Most of these elongation factors have been identified in multiple eukaryotes, and each appears to regulate a specific aspect of the overall elongation process. For example, the positive transcription elongation factor b (P-TEFb) increases the processivity of the elongating RNA pol II enzyme (Marshall and Price 1995; Price 2000). P-TEFb contains a cyclin-dependent protein kinase activity, Cdk9, that is required for this stimulation of the elongation process (Marshall *et al.* 1996; Mancebo *et al.* 1997). A second elongation factor, known as FACT, is required for the efficient elongation through chromatin templates *in vitro* (Orphanides *et al.* 1998). Nucleosomes constitute a physical barrier to the elongating polymerase and several factors, including FACT, appear to be required to overcome this barrier (Hartzog *et al.* 2002; Svejstrup 2002). Finally, the TFIIS protein is required for the movement of the polymerase through specific sites in the DNA template that can cause a pause and/or arrest in the elongation process (Fish and Kane 2002). The characterization of these, and other protein factors, has indicated that RNA pol II elongation is a highly conserved, complex process that is subject to regulation at multiple levels.

Recent work has shown that these elongation factors often work together to control specific stages of the elongation process. One example of this cooperation involves an additional elongation factor, the 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB)-sensitivity-inducing factor (DSIF; Wada *et al.* 1998a). DRB is a drug that blocks RNA pol II transcript elongation *in vitro* by inhibiting the protein kinase activity associated with P-TEFb (Tamm and Kikuchi 1979; Price 2000). One of the most interesting features of DSIF is that it

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appears to act as both a negative and positive regulator of RNA pol II elongation. In the early stages of elongation, DSIF binds to the RNA pol II enzyme and effectively blocks mRNA transcript elongation (Wada *et al.* 1998a). This block is alleviated by P-TEFb, which phosphorylates both the C-terminal domain (CTD) of the largest subunit of RNA pol II and one of the subunits of the DSIF complex (Wada *et al.* 1998b; Ivanov *et al.* 2000; Price 2000; Kim and Sharp 2001). The FACT elongation factor also seems to play a role in reversing the DSIF-mediated inhibition of elongation (Wada *et al.* 2000). Following the pTEFb-mediated phosphorylation, DSIF remains associated with the elongating polymerase and can apparently stimulate the elongation process (Ping and Rana 1999, 2001).

The DSIF elongation factor consists of two polypeptides that are the human homologs of the *Saccharomyces cerevisiae* Spt4p and Spt5p proteins (Wada *et al.* 1998a). The yeast *SPT4* and *SPT5* genes were originally identified in a genetic selection for mutations that suppressed the effects of specific promoter insertion mutations (Winston *et al.* 1984; Winston and Carlson 1992). These early studies suggested that Spt4p and Spt5p functioned together to influence RNA pol II transcription possibly by affecting chromatin structure (Swanson and Winston 1992; Hartzog *et al.* 2002). More recent work has indicated that these yeast proteins are indeed found within a single complex and that this Spt4p/Spt5p complex is likely regulating RNA pol II transcript elongation *in vivo* (Hartzog *et al.* 1998, 2002; Lindstrom and Hartzog 2001; Pokholok *et al.* 2002). Moreover, studies of the *Drosophila melanogaster* Spt5p homolog are consistent with this protein also playing a role in RNA pol II transcript elongation (Andrulis *et al.* 2000; Kaplan *et al.* 2000). Thus, the DSIF elongation factor appears to be a highly conserved protein complex that has both positive and negative effects on RNA pol II elongation.

The eukaryotic *RAS* genes encode small GTP-binding proteins that are key regulators of such fundamental processes as cell proliferation and differentiation (Marshall 1999; Shields *et al.* 2000). Ras proteins typically function as signaling switches that oscillate between active GTP-bound and inactive GDP-bound states (Broach 1991; Lowy and Willumsen 1993). In *S. cerevisiae*, two Ras proteins, Ras1p and Ras2p, together regulate the activity of the cAMP-dependent protein kinase (PKA; Kataoka *et al.* 1984; Toda *et al.* 1985). This Ras/PKA signaling pathway is a key regulator of cell growth and proliferation in this budding yeast (Broach 1991; Herman 2002). Recent work has indicated that these effects on growth are due, in part, to the regulation of RNA pol II activity (Chang *et al.* 2001; Howard *et al.* 2001, 2002; Herman 2002). In particular, these data suggest that at least one target of the Ras/PKA signaling pathway is associated with the CTD of Rpb1p, the largest subunit of RNA pol II (Herman 2002; Howard *et al.* 2002). This CTD is a highly conserved, repetitive structure that is a key site of regulation for multiple steps during the production of a mature mRNA (Greenleaf 1993; Hirose and Manley 2000). Although these data indicated that Ras/PKA activity was regulating an activity important for RNA pol II transcription, it was not clear what step of the transcription cycle was being targeted by this signaling pathway.

In this study, we present evidence suggesting that the Ras/PKA signaling pathway regulates the elongation step of the RNA pol II transcription process. In particular, the data suggest that Ras/PKA activity targets the Spt4p/Spt5p elongation factor. This regulation may be direct as Spt5p is an efficient *in vitro* substrate for PKA. Altogether, the data indicate that RNA pol II transcript elongation, like initiation, may be subject to regulation by signal transduction pathways that control the cellular response to changes in the extracellular environment.

MATERIALS AND METHODS

Growth media: Standard *Escherichia coli* growth conditions and media were used throughout this study (Miller 1972). YM glucose yeast medium consists of 0.67% yeast nitrogenous base (Difco, Detroit), 2% glucose, and all growth supplements required for cell proliferation. YPAD rich growth medium consists of 1% yeast extract (Difco), 2% Bacto-peptone (Difco), 50 mg/liter adenine-HCl, and 2% glucose. 6-Azauracil (6AU) and mycophenolic acid (MPA) (Sigma, St. Louis) were added to the growth media at the concentrations specified.

Plasmids: The plasmids pPHY453, pJR1040, and pJR1052 consist of the *RAS2^{val19}* allele cloned into pRS415, pRS416, and pRS414, respectively. The pRS plasmids were described previously (Sikorski and Hieter 1989). The *LEU2*-marked *MET3-RAS2^{val19}* plasmid, pPHY795, was constructed as described (Howard *et al.* 2002). The high-copy *PDE2* plasmid, pPHY1299, was constructed by subcloning a 2.4-kb *Bam*HI-*Hin*dIII fragment that contains *PDE2* into pRS425. The high-copy *IMD2* plasmid, pPHY1410, was constructed by subcloning the *Xho*I-*Not*I fragment containing *IMD2* from pPHY1401 into pRS425; pPHY1401 was provided by Daniel Reines and is a pRS426-based plasmid that contains *IMD2*. The pGH31 plasmid encodes a Myc-tagged version of Spt5p and was provided by Grant Hartzog.

The Spt5p expression plasmids used in the *in vitro* kinase assay were kindly provided by Grant Hartzog. The expression plasmid, pGH11, encodes a hemagglutinin (HA) epitope-tagged version of Spt5p that includes amino acids 12–1063. This HA-Spt5p construct is under the control of the galactose-inducible promoter from the *GAL1* gene. The plasmid, pJG4-6, is a control vector that lacks *SPT5* sequences.

Yeast strain constructions and genetic methods: The strains used in this study are listed in Table 1. Unless otherwise noted, 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).

To test for genetic interactions with the *RAS2^{val19}* allele, strains were transformed with either pPHY453 or pPHY795 (*MET3-RAS2^{val19}*). The pPHY795 transformants were recovered on media containing 500 μM methionine and then grown on medium lacking methionine to induce expression of *RAS2^{val19}*.

The *rpb1-104* strain, PHY2857, was constructed by a plasmid shuffle procedure. The starting strain for this procedure, PHY2851, contains a chromosomal deletion of *RPB1* and a plasmid bearing the *rpo21-18* allele. This strain was trans-

TABLE 1

Yeast strains used in this study

Strain	Genotype	Alias	Source/Reference
AHJ2	*MAT***a** *leu2Δ1 his3Δ200 ura3-52 ctk1Δ::HIS3*	PHY1872	Lee and Greenleaf (1991)
DY5393	*MAT***a** *spt16 (G132D) ade2 can1 his3 leu2 trp1 ura3*	PHY3599	David Stillman
FY267	*MAT*α *his4-912δ lys2-128δ leu2Δ1*	PHY2500	Hartzog *et al.* (1998)
FY1646	*MAT*α *his4-912δ lys2-128δ leu2Δ1 spt4Δ2::HIS3*	PHY2501	Hartzog *et al.* (1998)
FY1645	*MAT*α *his4-912δ lys2-128δ leu2Δ1 spt5-242*	PHY2503	Hartzog *et al.* (1998)
FY1655	*MAT*α *his4-912δ lys2-128δ leu2Δ1 spt6-14*	PHY2504	Hartzog *et al.* (1998)
FY1667	*MAT*α *his4-912δ lys2-128δ leu2Δ1 spt6-50*	PHY2505	Hartzog *et al.* (1998)
KY459	*MAT*α *his3Δ200 lys2-173R2 leu2Δ1 ura3-52 ade8 rtf1Δ100::URA3*	PHY2579	Costa and Arndt (2000)
GHY285	*MAT*α *his3Δ200 lys2-173R2 leu2Δ1 ura3-52 ppr2Δ100::URA3-hisG*	PHY2580	Costa and Arndt (2000)
GHY379	*MAT*α *his4-912δ lys2-128δ leu2Δ1 spt5-194*	PHY2593	Hartzog *et al.* (1998)
GHY366	*MAT*α *his4-912δ lys2-128δ leu2Δ1 spt5-276*	PHY2595	Hartzog *et al.* (1998)
GHY149	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52 rpb1-244*	PHY2596	Hartzog *et al.* (1998)
GHY190	*MAT***a** *his4-912δ lys2-128δ leu2Δ1 ura3-52 rpb1-221*	PHY2597	Hartzog *et al.* (1998)
FY124	*MAT*α *leu2Δ1 ura3-52 trp1Δ63*	PHY2812	Fred Winston
PHY1942	*MAT*α *his3Δ200 leu2-3,112 lys2-801 prb1::hisG prc1::HIS3 pep4::LEU2 suc2-Δ9 trp1-101 ura3-52*		
PHY2848	*MAT*α *his3Δ200 leu2Δ1trp1Δ63 lys2-128δ rpb1Δ187::HIS3* p(*RPB1/LEU2*)		
PHY2850	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 lys2-128δ rpb2Δ297::HIS3* p(*rpb2-10/URA3*)		
PHY2851	*MAT*α *his3Δ200 leu2Δ1trp1Δ63 lys2-128δ ura3-52 rpb1Δ187::HIS3* p(*rpo21-18/TRP1*)		
PHY2857	*MAT*α *his3Δ200 leu2Δ1trp1Δ63 lys2-128δ ura3-52 rpb1Δ187::HIS3* pPHY854		
PHY2193	*MAT***a** *his3Δ200 leu2-3,112 ura3-52 rpb1Δ187::HIS3* p(*rpb1-104/LEU2*) pRS416		Howard *et al.* (2002)
PHY2194	*MAT***a** *his3Δ200 leu2-3,112 ura3-52 rpb1Δ187::HIS3* p(*RPB1/LEU2*) pJR1040		Howard *et al.* (2002)
PHY2195	*MAT***a** *his3Δ200 leu2-3,112 ura3-52 rpb1Δ187::HIS3* p(*RPB1/LEU2*) pRS416		Howard *et al.* (2002)
PHY1025	*MAT*α *lys2Δ his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*		Herman and Rine (1997)
YJJ662	*MAT***a** *leu2Δ1 his3Δ200 ura3-52*	PHY1871	Lee and Greenleaf (1991)

formed with a *LEU2*-marked *rpb1-104* plasmid, pPHY854, and grown under conditions favoring the loss of the *TRP1*-marked *rpo21-18* plasmid. Cells that had lost this latter plasmid were identified by their failure to grow on plates lacking tryptophan.

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). The probes were labeled with the Prime-It II random primer labeling kit (Stratagene, La Jolla, CA).

Immunoprecipitation and *in vitro* kinase assays: For Spt5p, wild-type cells containing either a plasmid encoding HA-Spt5p (pGH11) or a control vector (pJG4-6) were grown to midlogarithmic phase in YM glucose minimal medium and transferred to YM minimal medium with 2% raffinose for 12 hr. The cells were then transferred to YM minimal medium containing 2% raffinose and 5% galactose and incubated for 2.5 hr at 30°. The cells were collected by centrifugation, spheroplasted with 0.1 mg/OD_{600} Zymolyase-20T (Seikagaku, Rockville, MD), and lysed by the addition of an excess of ice-cold TBS (25 mm Tris-HCl, pH 7.4, 140 mM NaCl). Cell lysates were incubated overnight at 4° with 50 μl of agarose beads that were conjugated to an antibody specific for the HA epitope (Roche, Indianapolis). The beads were washed with TBS and resuspended in kinase reaction buffer (10 mm $MgCl_2$, 4.5 mm dithiothreitol, 5 mm NaF, 50 mm KP*i*, pH 7.15) containing 1 μCi [γ-^{32}P]ATP (Perkin-Elmer, Norwalk, CT) and 10 units of bovine PKA (Sigma). The reactions were incubated for 30 min at 25°, and the beads were washed several times with PBS. Bound proteins were eluted and separated in a 7.5% SDS-polyacrylamide gel. The gel was fixed with a 10% trichloroacetic acid/10% acetic acid/50% methanol solution, dried, and exposed to X-ray film.

For Western immunoblot analysis, the immunoprecipitated proteins were separated in a 7.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Hybond ECL; Amersham, Arlington Heights, IL). The membrane was hybridized with a 1:1000 dilution of a rat antibody specific for the HA epitope (Roche), followed by a 1:10,000 dilution of a horseradish peroxidase-antibody conjugate specific for rat IgG (Sigma). The supersignal chemiluminescent substrate (Pierce, Rockford, IL) was subsequently used to illuminate the reactive bands.

A similar protocol was used for the Ppr2p (TFIIS) experiments with the following modifications. PHY1942 cells were grown to midlog phase in YPAD medium and cell lysates were prepared from eight OD_{600} unit equivalents of cells. The lysates were incubated overnight at 4° with anti-TFIIS rabbit antibody, kindly provided by Caroline Kane, at a 1:1000 dilution. The immunoprecipitates were then collected on Protein A-Sepharose beads and washed as described above. For the Western immunoblotting experiments, the nitrocellulose membrane was incubated with a 1:10,000 dilution of the anti-TFIIS rabbit antibody, followed by a 1:3000 dilution of a horseradish peroxidase-antibody conjugate specific for rabbit IgG (Amersham).

TABLE 2

***RAS2*^{val19} mutants were very sensitive to drugs that inhibit the growth of mutants defective for RNA polymerase II transcript elongation**

	No drug	6AU	MPA
Wild type	++	++	+
RAS2^{val19}	++	−	−
*spt4*Δ	++	−	−
spt5-194	++	+/−	+/−
spt5-242	++	++	+
spt5-276	++	++	+
spt6-14	++	++	+
spt6-50	++	+/−	+/−
*rtf1*Δ	++	−	−
*ppr2*Δ	++	−	−
ras2-23	++	++	+

The relative growth rates of the indicated yeast strains were assessed following 3–4 days of growth at 30° on minimal media containing either 125 μg/ml 6AU or 30 μg/ml MPA.

RESULTS

***RAS2*^{val19} cells were sensitive to drugs that inhibit the growth of mutants defective for RNA polymerase II transcript elongation:** Our previous work suggested that the Ras/PKA pathway influences RNA polymerase II transcription by regulating the activities of proteins associated with the Rpb1p CTD (Chang *et al.* 2001; Howard *et al.* 2002). However, this work did not identify the specific step affected by Ras/PKA activity. Here, we tested whether this control might be exerted at the level of transcript elongation by examining the sensitivity of particular Ras pathway mutants to two drugs, 6AU and MPA. Both of these drugs inhibit the activity of IMP dehydrogenase (IMPDH), the rate-limiting enzyme in the *de novo* synthesis of GTP, and thus result in lower intracellular levels of GTP (Exinger and Lacroute 1992). Previous studies have shown that low concentrations of GTP cause RNA polymerase molecules to stall and arrest more frequently during the elongation process *in vitro* (Powell and Reines 1996; Uptain *et al.* 1997). In addition, both 6AU and MPA cause growth defects in yeast mutants defective for RNA polymerase II transcript elongation (Archambault *et al.* 1992; Nakanishi *et al.* 1995; Powell and Reines 1996; Costa and Arndt 2000; Squazzo *et al.* 2002). Thus, an increased sensitivity to these drugs can be an indication of an underlying defect in RNA polymerase II elongation.

We tested the effects of 6AU and MPA on the growth of a variety of mutants that affect the Ras signaling pathway. Interestingly, mutants with elevated levels of Ras signaling activity, like *RAS2*^{val19}, were found to be as sensitive to both 6AU and MPA as any of the previously described elongation mutants (Table 2). The *RAS2*^{val19} allele encodes a hyperactive form of Ras2p and results in constitutively high levels of Ras signaling activity (Kataoka *et al.* 1984). The growth defects for three of the most sensitive mutants examined, *RAS2*^{val19}, *ppr2*Δ, and *spt4*Δ, are shown in Figure 1. In contrast to these results with *RAS2*^{val19}, we found that mutants with diminished levels of Ras signaling activity were not significantly affected by the concentrations of 6AU and MPA used in this study (data not shown). For example, the growth of a temperature-sensitive *ras2-23 ras1*Δ double mutant was not inhibited by the presence of either drug in the growth medium (Table 2). Therefore, elevated levels of Ras signaling activity resulted in an increased sensitivity to the drugs 6AU and MPA.

Although the *S. cerevisiae* Ras proteins function through multiple effectors, our previous work had indicated that the cAMP/PKA effector pathway was the most important for the Ras pathway effects on RNA pol II transcription (Chang *et al.* 2001; Howard *et al.* 2001, 2002). To test whether this PKA pathway was also responsible for the drug sensitivities observed here, a high-copy plasmid containing the *PDE2* gene was introduced into a *RAS2*^{val19} mutant. *PDE2* encodes a high-affinity cAMP phosphodiesterase that, when overproduced, results in lowered levels of PKA activity (Sass *et al.* 1986). We found that this *PDE2* plasmid suppressed the 6AU sensitivity of *RAS2*^{val19} mutants (data not shown). Therefore, these data are consistent with a role for the Ras/PKA signaling pathway in the regulation of RNA polymerase II transcript elongation.

Elevated levels of Ras/PKA signaling were lethal in a specific subset of mutants defective for RNA polymerase II transcript elongation: Recent studies have shown that the drugs 6AU and MPA also affect the growth of a number of yeast mutants that are not obviously defective for RNA pol II elongation (Padilla *et al.* 1998; Desmoucelles *et al.* 2002). We therefore carried out a detailed genetic analysis in an attempt to test the assertion that the Ras/PKA pathway was influencing RNA pol II transcript elongation. Specifically, we asked whether increasing the levels of Ras/PKA signaling activity would enhance or suppress defects associated with mutants that were sensitive to 6AU and MPA. Interestingly, this analysis found that the elevated levels of Ras/PKA activity associated with the *RAS2*^{val19} allele resulted in a severe growth defect in a particular subset of mutants defective for RNA polymerase II transcript elongation. In contrast, the presence of *RAS2*^{val19} did not affect the growth rate of the 6AU/MPA-sensitive mutants tested that were not defective for RNA pol II elongation (data not shown; Howard *et al.* 2002). These latter mutants included those defective for vacuolar functions (*vps15*, *vps33*), chromatin remodeling activities (*snf2*, *snf6*), and protein secretion (*sec22*). For most of these experiments, the *RAS2*^{val19} coding sequences were fused to the inducible promoter from the *MET3* gene (Howard *et al.* 2001). This promoter is active in cells grown in media lacking methionine and is repressed by the presence of

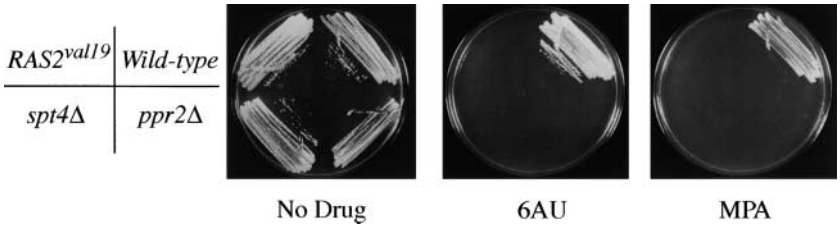
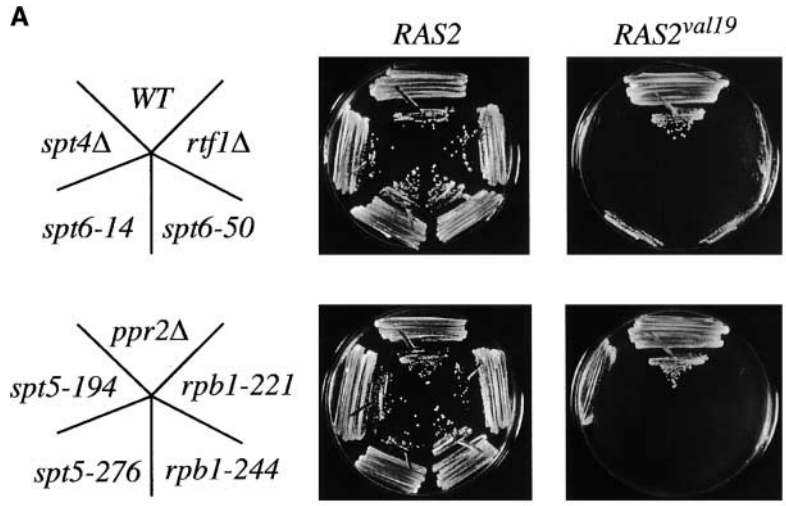


FIGURE 1.—*RAS2*^{val19} cells were sensitive to 6AU and MPA, two drugs that lower intracellular GTP levels. Strains with the indicated genotypes were grown on YM glucose minimal medium supplemented with no drug, 100 μg/ml 6AU, or 30 μg/ml MPA and incubated for 3 days at 30°. The strains analyzed were wild type (FY267), *RAS2*^{val19} (FY267 carrying the *RAS2*^{val19} plasmid, pPHY453), *spt4*Δ (FY1646), and *ppr2*Δ (GHY285). All strains, except the *RAS2*^{val19} mutant, were carrying the control vector, pRS415.

methionine in the growth medium (CHEREST *et al.* 1987; MOUNTAIN *et al.* 1991; HOWARD *et al.* 2002).

The expression of *RAS2*^{val19} resulted in a strong synthetic growth defect in the *spt4*, *spt5*, and *spt6* mutants (Figure 2). In addition, *RAS2*^{val19} was synthetically lethal with several other mutations that compromise transcript elongation. These mutations included disruptions of the genes encoding Rtf1p, a component of the Paf1 complex (STOLINSKI *et al.* 1997; COSTA and ARNDT 2000), and Ctk1p, a Rpb1p CTD kinase important for transcript elongation (JONA *et al.* 2001; Figure 2). In addition, *RAS2*^{val19} was synthetically lethal with two particular alleles of *RPB1*, *rpb1-221* and *rpb1-224* (Figure 2). These *RPB1* alleles were isolated in a genetic selection for mutations that suppressed specific *spt5* mutations (HARTZOG *et al.* 1998). Both *rpb1-221* and *rpb1-244* mutants are sensitive to 6AU and it has been suggested that these mutants are defective in RNA pol II transcript elongation (HARTZOG *et al.* 1998). It is important to point out that other *rpb1* alleles that do not confer a sensitivity to 6AU, such as *rpb1-1* and *rpb1-5*, do not exhibit a genetic interaction with *RAS2*^{val19} (HOWARD *et al.* 2002).

Interestingly, not all mutations thought to compromise transcript elongation were affected by the presence of *RAS2*^{val19} (Figure 2). For example, *RAS2*^{val19} was not synthetically lethal with mutations that affect the activity of the elongation factor, TFIIS. These mutations included a disruption of *PPR2*, the gene encoding the *S. cerevisiae* TFIIS, and *rpo21-18*, an allele of *RPB1* that lowers TFIIS activity by disrupting the Ppr2p-Rpb1p association (WU *et al.* 1996; UPTAIN *et al.* 1997; REINES *et al.* 1999; WIND and REINES 2000). A third mutation that was insensitive to *RAS2*^{val19} was *rpb2-10*, a mutation in the gene encoding Rpb2p, the second largest subunit of RNA pol II. The *rpb2-10* mutation has been shown to compromise transcript elongation and to cause phenotypes similar to those seen with *ppr2*Δ and *rpo21-18* (SHAW and REINES 2000; SHAW *et al.* 2001). Finally, several *spt16* mutants,



B

	RAS2	RAS2^{val19}
Wild-type	++	++
spt4Δ	++	-
spt5-194	++	+/-
spt5-242	++	-
spt5-276	++	-
spt6-14	++	+/-
spt6-50	++	+/-
rtf1Δ	++	-
rpb1-221	++	-
rpb1-244	++	-
rpo21-18	++	++
rpb2-10	++	++
ppr2Δ	++	++
spt16	++	++
ctk1Δ	++	-

FIGURE 2.—Genetic interactions between the Ras/PKA signaling pathway and the RNA pol II elongation machinery. (A) Elevated levels of Ras/PKA activity caused growth defects in a specific subset of elongation mutants. Strains with the indicated genotype were grown on YM glucose minimal medium for 3 days at 30°. The strains all carried a plasmid, pPHY795, that contains the *MET3-RAS2*^{val19} allele and were grown under conditions in which the *MET3* promoter was either repressed (*RAS2*; 500 μM methionine) or active (*RAS2*^{val19}; 0 μM methionine). The strains analyzed were wild type (FY267), *spt4*Δ (FY1646), *spt5-194* (GHY379), *spt5-276* (GHY366), *spt6-14* (FY1655), *spt6-50* (FY1667), *rtf1*Δ (KY459), *rpb1-221* (GHY190), *rpb1-244* (GHY149), and *ppr2*Δ (GHY285). (B) The relative growth rate of the indicated strains containing either a wild-type (*RAS2*) or hyperactive (*RAS2*^{val19}) allele of the *RAS2* locus was assessed after 3 days of growth on minimal medium at 30°. For the *RAS2*^{val19} experiments, strains were carrying the *MET3-RAS2*^{val19} allele and were grown in medium lacking methionine to allow for the expression of *RAS2*^{val19}.

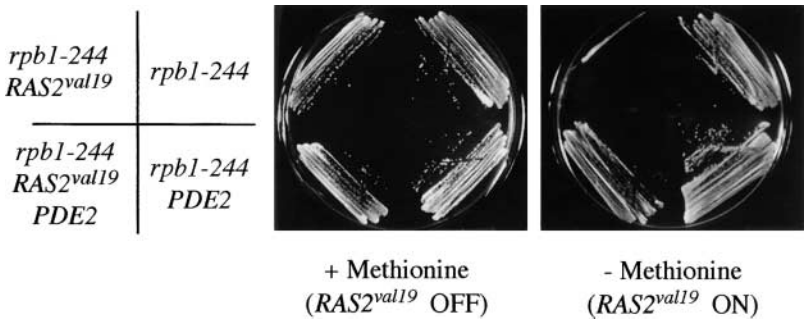


FIGURE 3.—The *RAS2*^{val19} effects on mutants defective for RNA pol II transcript elongation were mediated by the cAMP-PKA effector pathway. The *rpb1-244* strain, PHY2596, carrying either a *MET3-RAS2*^{val19} plasmid (pPHY796) or a control vector (pRS416), was transformed with a high-copy plasmid bearing *PDE2* (pPHY1299) or the control vector, pRS425. The resulting strains were streaked to YM glucose minimal medium containing either 500 μM (*RAS2*^{val19} OFF) or 0 μM methionine (*RAS2*^{val19} ON) and incubated for 3 days at 30°.

including *spt16-1* and *spt16-11*, were found to be insensitive to elevated levels of Ras/PKA activity (Figure 2; FORMOSA *et al.* 2001). Spt16p is a subunit of a yeast complex that is thought to be the functional equivalent of the mammalian FACT elongation factor (ORPHANIDES *et al.* 1999). Thus, the elongation mutants tested could be divided into two classes on the basis of their relative sensitivity to the activity of the Ras signaling pathway.

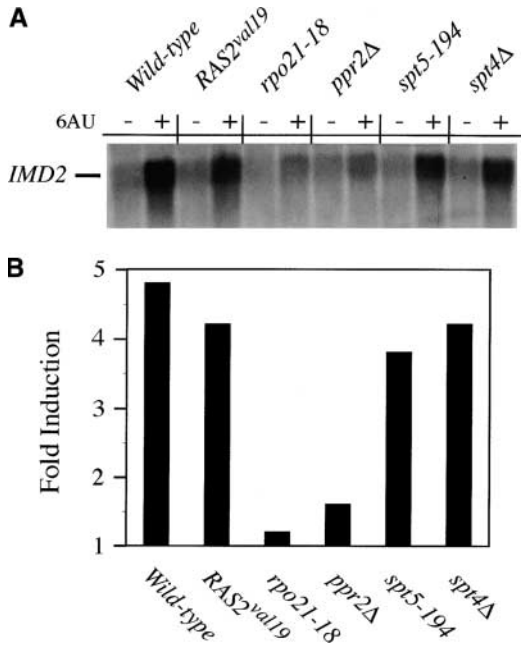


FIGURE 4.—*RAS2*^{val19} and *spt* mutants were not defective in *IMD2* induction following exposure to 6AU. (A) Northern blot analyses of *IMD2* mRNA levels. Strains with the indicated genotype were grown in YM glucose minimal medium to mid-log phase at 30°, and the cultures were then split in half. 6AU was added to one-half of each culture to a final concentration of 100 μg/ml, and the cultures were incubated at 30° for an additional 3 hr. Total RNA was prepared as described in MATERIALS AND METHODS, and the levels of *IMD2* mRNA were assessed by a Northern blot analysis. (B) Phosphorimager quantification of the Northern blot data presented in A. Fold induction refers to the ratio of the amount of *IMD2* mRNA in the 6AU-containing culture to that found in the control culture. The relative amount of *IMD2* mRNA was normalized to that of the loading control, *ACT1*; similar levels of *ACT1* mRNA were found in all samples. The data shown are representative of at least three independent experiments. The strains analyzed were wild type (FY267), *RAS2*^{val19} (FY267 carrying the *RAS2*^{val19} plasmid, pPHY453), *rpo21-18* (PHY2851), *ppr2*Δ (GHY285), *spt5-194* (GHY379), and *spt4*Δ (FY1646). All strains, except the *RAS2*^{val19} mutant, were carrying the control vector, pRS415.

These genetic interactions with *RAS2*^{val19} were apparently due to increased levels of signaling through the PKA effector pathway as the growth defects were effectively suppressed by a high-copy plasmid containing *PDE2*. For example, the synthetic lethality associated with the *RAS2*^{val19} *rpb1-244* double mutant was suppressed by the presence of this *PDE2* plasmid (Figure 3). Thus, elevated levels of Ras/PKA signaling activity specifically inhibited the growth of a subset of mutants impaired for transcript elongation.

***The RAS2*^{val19} and *spt* mutants were not defective for the induction of *IMD2*:** Previous work has suggested that the 6AU- and MPA-sensitive phenotypes of *ppr2*Δ, *rpo21-18*, and *rpb2-10* cells are due, at least in part, to the inability of these mutants to induce transcription from one of the IMPDH genes, *IMD2* (SHAW and REINES 2000). In wild-type cells, the presence of either 6AU or MPA results in a 3- to 5-fold increase in the levels of *IMD2* mRNA (Figure 4). This induction of *IMD2* does not occur in *ppr2*Δ, *rpo21-18*, and *rpb2-10* mutants (SHAW *et al.* 2001). We tested whether *RAS2*^{val19} and the drug-sensitive *spt4*Δ and *spt5-194* mutants were similarly defective in inducing *IMD2* transcription following an exposure to 6AU or MPA. Interestingly, we found that the *RAS2*^{val19} and *spt* mutants were able to induce *IMD2* to levels that approached those seen in wild-type cells. For example, after a 3-hr treatment with 6AU, *IMD2* expression in *RAS2*^{val19} and *spt* mutants was 4.2-fold above the basal level, only slightly lower than the 4.8-fold induction observed in wild-type cells (Figure 4). In contrast, this induction of *IMD2* was greatly attenuated in *ppr2*Δ and *rpo21-18* mutants (Figure 4) and to a lesser degree in *rtf1*Δ mutants (data not shown; SQUAZZO *et al.* 2002). Very similar results were obtained following exposure of these elongation mutants to MPA. Again, after a 3-hr exposure to MPA, we found that *RAS2*^{val19} and *spt* mutants induced *IMD2* expression to an almost wild-type level (Figure 5). This differed dramatically from the MPA effects on the *ppr2*Δ and *rpo21-18* mutants. In these latter mutants, treatment with MPA resulted in a slight decrease in the levels of *IMD2* mRNA.

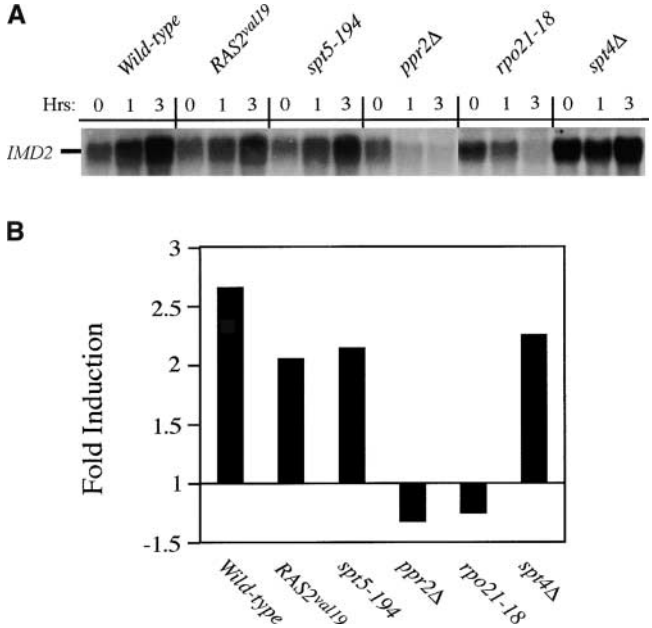


FIGURE 5.—*RAS2*^{val19} and *spt* mutants were not defective in *IMD2* induction following exposure to MPA. (A) Northern blot analyses of *IMD2* mRNA levels. Strains with the indicated genotype were grown in YM glucose minimal medium to mid-log phase at 30°. One-third of the culture was removed and used as the 0-hr control. MPA was added to the remaining two-thirds of the culture to a final concentration of 30 μg/ml. These cultures were then incubated for an additional 1 or 3 hr at 30°. Total RNA was then prepared from each of the three aliquots, and the levels of *IMD2* were assessed by a Northern blot analysis. (B) Phosphorimager quantification of the Northern blot data presented in A. Fold induction refers to the ratio of the amount of *IMD2* mRNA in the MPA-containing cultures after 1 hr to that found in the control, or 0-hr, culture. The relative amount of *IMD2* mRNA was normalized to that of the loading control, *ACT1*. The data shown are representative of at least three independent experiments. Note that the 0-hr time point for the *spt4*Δ strain was overloaded and that this was corrected for during the *ACT1* normalization process. The strains analyzed are listed in the Figure 4 legend.

These data suggest that there are two general classes of drug-sensitive elongation mutants: those that are strikingly defective in *IMD2* induction and those that are not. The *ppr2*Δ, *rpb2-10*, *rpo21-18*, and *rtf1*Δ mutants fall into the former class, whereas *spt4*Δ and *spt5-194* mutants can be placed into the latter. Interestingly, these molecular data established a functional link between the *RAS2*^{val19} and *spt* mutants and were generally consistent with the genetic interactions observed above. In particular, *RAS2*^{val19} was synthetically lethal with the *spt* mutations that fell into the latter class of elongation mutant. Elevated levels of Ras/PKA signaling did not have any significant effect on the growth of *ppr2*Δ, *rpo21-18*, or *rpb2-10* mutants. This correlation was not absolute, however, as the *rtf1*Δ mutant exhibited a synthetic growth defect with *RAS2*^{val19}, but was defective for the induction of *IMD2* mRNA.

A final point worth noting is that these data challenge a current model proposing that the sensitivity of elongation mutants to 6AU and MPA is a direct consequence of a failure to induce *IMD2* mRNA (SHAW *et al.* 2001). This correlation was not upheld in these present studies. For example, the *spt4*Δ mutant was very sensitive to both of these drugs but yet did not exhibit a significant defect in *IMD2* expression. Instead, these data indicate that, at least for some elongation mutants, the sensitivity to IMPDH inhibitors is not a direct result of a failure to induce *IMD2* transcription. The precise reason for their sensitivity to these drugs remains to be uncovered.

Truncation of the Rpb1p CTD resulted in a sensitivity to 6AU and MPA: Previous work from our lab identified a functional interaction between the Ras/PKA signaling pathway and the Rpb1p CTD (HOWARD *et al.* 2002). In addition, other studies have demonstrated a connection between this CTD and the Spt4p/Spt5p elongation factor (LINDSTROM and HARTZOG 2001). In particular, *RAS2*^{val19}, *spt4*, and *spt5* mutations all exhibit synthetic growth defects with mutations that lower the activity of the CTD kinases Kin28p and Ctk1p and with mutations that truncate the Rpb1p CTD (Figure 6A). The data presented here close this circle and identify genetic interactions between *RAS2*^{val19} and these *spt* mutations. Altogether, these data suggest the existence of a functional relationship among the Ras/PKA signaling pathway, the Rpb1p CTD, and the Spt4p/Spt5p complex. Therefore, we tested whether mutations that truncate the Rpb1p CTD, like *rpb1-104*, would cause phenotypes similar to those observed with *RAS2*^{val19}, *spt4*, and *spt5* mutants. The *rpb1-104* mutant encodes an Rpb1p that has only 11 of the 27 heptameric repeat units found in the wild-type CTD (NONET and YOUNG 1989); the consensus heptad repeat is Y_1-S_2-P_3-T_4-S_5-P_6-S_7 (ALLISON *et al.* 1985; CORDEN *et al.* 1985). We found that the *rpb1-104* mutant was indeed very sensitive to both 6AU and MPA; the presence of either drug in the growth medium resulted in a severe growth defect (Figure 6B). In addition, as with the *RAS2*^{val19} and *spt* mutants, *rpb1-104* cells did not exhibit a defect in the induction of *IMD2* expression following a 3-hr exposure to either of these drugs (Figure 6, C and D). Therefore, on the basis of these phenotypes, we would place this *rpb1-104* mutant into the same class as the *RAS2*^{val19}, *spt4*, and *spt5* mutants.

Mutations in *SPT5* result in an inability to enter into a normal stationary phase: One possibility suggested by the above data is that the Spt4p/Spt5p complex could be a CTD-associated target of the Ras/PKA signaling pathway. Such a model would predict that mutants defective for this complex would exhibit phenotypes similar to those observed with *RAS2*^{val19} cells. The Ras/PKA pathway plays a central role in regulating growth in response to changes in nutrient availability. Cells with the *RAS2*^{val19} allele have constitutively elevated levels of Ras/PKA activity and are unable to adopt stationary phase characteristics following nutrient deprivation (KATAOKA *et al.* 1984; BROEK *et al.* 1985; TODA *et al.*

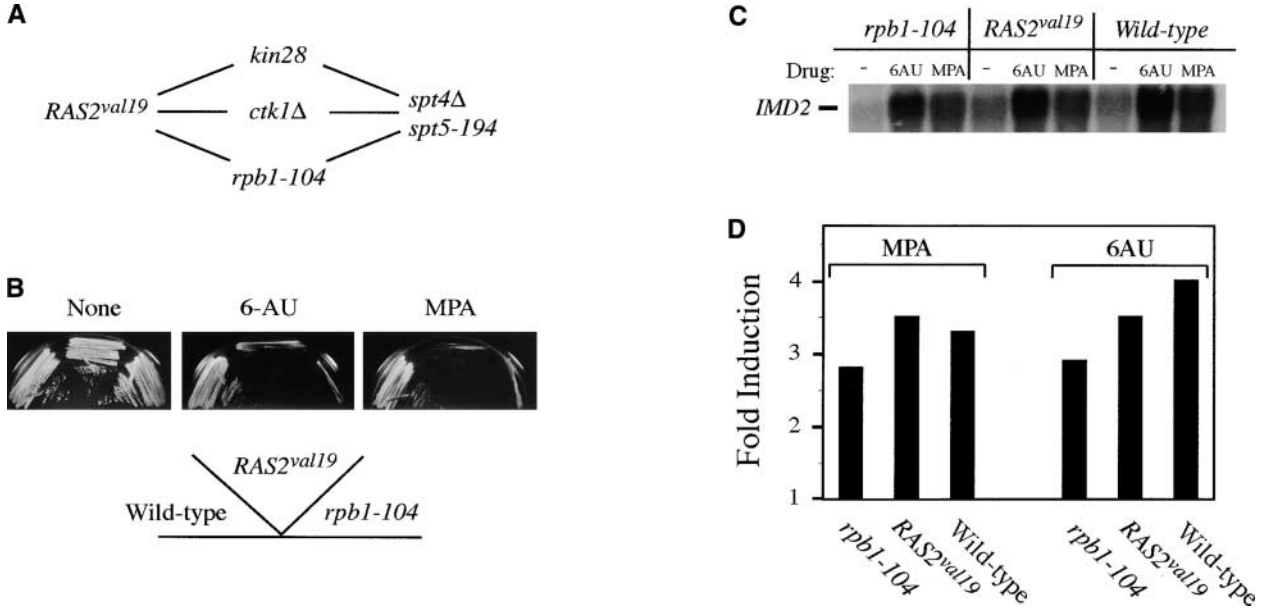


FIGURE 6.—Truncation of the Rpb1p CTD resulted in phenotypes similar to those observed with *RAS2*^{val19} and *spt* mutants. (A) *RAS2*^{val19} and *spt* mutations exhibited a similar set of genetic interactions with mutations affecting the Rpb1p CTD. The schematic shows the synthetic lethal interactions observed between the indicated mutations. The *RAS2*^{val19} genetic interactions were characterized either in this study or in previous work from our lab (HOWARD *et al.* 2002), and the *spt* interactions were identified elsewhere (LINDSTROM and HARTZOG 2001). (B) Truncation of the Rpb1p CTD resulted in sensitivity to both 6AU and MPA. Strains with the indicated genotype were grown for 3 days at 30° on YM glucose medium that contained no drug, 125 μg/ml 6AU, or 30 μg/ml MPA. The strains analyzed were wild type (PHY2195), *RAS2*^{val19} (PHY2194), and *rpb1-104* (PHY2193). (C) The *rpb1-104* CTD truncation mutant exhibited wild-type levels of *IMD2* mRNA following a challenge with either 6AU or MPA. Strains with the indicated genotype were grown in YM glucose minimal medium to midlog phase at 30° and the cultures were divided into three equal aliquots. The different aliquots then received no drug, 6AU (125 μg/ml), or MPA (30 μg/ml) and were incubated for an additional 3 hr at 30°. Total RNA was prepared from each of the three aliquots, and the levels of *IMD2* were assessed by a Northern blot analysis. The strains analyzed were as described in B. (D) Phosphorimager quantification of the Northern blot data presented in C. Fold induction refers to the ratio of the amount of *IMD2* mRNA in the drug-containing cultures to that found in the no drug control. In all cases, the relative amount of *IMD2* mRNA was normalized to that of the loading control, *ACT1*.

1985; HERMAN 2002). As a result, such cells rapidly lose viability under nutrient-limiting conditions. An example of this defect is shown in Figure 7, where the *RAS2*^{val19} culture has at least 3000-fold fewer survivors relative to the wild type after 9 days of growth in minimal medium. Interestingly, the *spt5-194* mutant also exhibited a dramatic loss of viability under these growth conditions (Figure 7). A similar defect in stationary phase survival has been reported for the CTD truncation mutant, *rpb1-104* (HOWARD *et al.* 2002). The *spt5-194* mutant was also defective for a second stationary phase phenotype. Wild-type cells typically accumulate elevated levels of the storage carbohydrate, glycogen, during stationary phase growth. We found that *spt5-194* cells, like both the *RAS2*^{val19} and *rpb1-104* mutants, failed to accumulate normal levels of glycogen following nutrient deprivation (data not shown; HOWARD *et al.* 2002). Therefore, Spt5p function is required for the entry into a normal stationary phase.

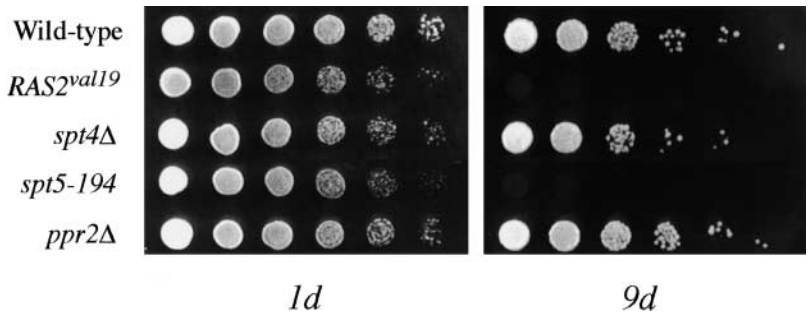


FIGURE 7.—*RAS2*^{val19} and *spt5* mutants exhibited defects in stationary phase viability. Strains with the indicated genotypes were grown for either 1 or 9 days in YM glucose minimal medium at 30°. Fivefold serial dilutions of these cultures were then spotted to a solid medium and incubated for 4 days at 30°. The number of colonies formed after this incubation was a measure of the number of survivors in the original stationary phase cultures. The strains analyzed were wild type (FY267), *RAS2*^{val19} (FY267 with pPHY453), *spt4*Δ (FY1646), *spt5-194* (GHY379), and *ppr2*Δ (GHY285). All of the strains except the *RAS2*^{val19} derivative contained the control vector, pRS415.

It is important to point out that the other elongation mutants tested did not exhibit these stationary phase viability defects (Figure 7 and data not shown). This included strains in the other mutant class, like *ppr2*Δ, that were defective for the induction of *IMD2* expression. The *ppr2*Δ stationary phase cultures contained essentially the same number of survivors after 9 days of growth as the wild-type strain (Figure 7). In addition, the *spt4*Δ mutant did not exhibit defects in either cell survival or glycogen accumulation during the stationary phase of growth (Figure 7 and data not shown). Therefore, the *spt5* mutants were unique among the elongation mutants with respect to these stationary phase defects.

Spt5p was phosphorylated by PKA *in vitro*: Several of the above observations were consistent with a model proposing that the Ras/PKA pathway regulates RNA pol II transcription by targeting Spt5p within the Spt4p/Spt5p elongation factor complex. First, the responses of *spt4* and *spt5* mutants to the drugs 6AU and MPA were very similar to those exhibited by the *RAS2*^{val19} mutant. Second, *spt5* mutants were the only elongation-defective mutants that exhibited stationary phase-specific phenotypes similar to those observed with *RAS2*^{val19}. Third, *RAS2*^{val19} caused a severe growth defect in a *spt4* null mutant, a strain that does not contain any Spt4p. Thus, Spt4p could not be the Ras/PKA target responsible for the growth defects observed in this study. Instead, the data suggested that Spt5p might be the relevant substrate of PKA and this possibility was examined here with an *in vitro* phosphorylation assay. For these experiments, Spt5p, and other potential targets, were immunoprecipitated from cell extracts and then incubated with [γ-^{32}P]ATP in the presence, or absence, of the PKA enzyme. Spt5p was found to be efficiently phosphorylated in a PKA-dependent manner in this assay system (Figure 8A). Two Spt5p-specific bands were identified in the cell extracts and both were able to serve as *in vitro* substrates for PKA. In contrast, we have found that most proteins tested in this assay are not able to serve as substrates for PKA; proteins that are phosphorylated have generally been shown to be *in vivo* targets of PKA. One example of a protein that is not phosphorylated in this assay system, Ppr2p, the *S. cerevisiae* TFIIS protein, is shown in Figure 8B. Although the observed phosphorylation of Spt5p could have been due to a PKA-activated protein kinase that was present in the immunoprecipitates, the intensity of the phosphorylation signal was more consistent with Spt5p being a direct target for PKA. Thus, these biochemical data support the proposition that the Spt4p/Spt5p complex is a direct target of the Ras/PKA signaling pathway.

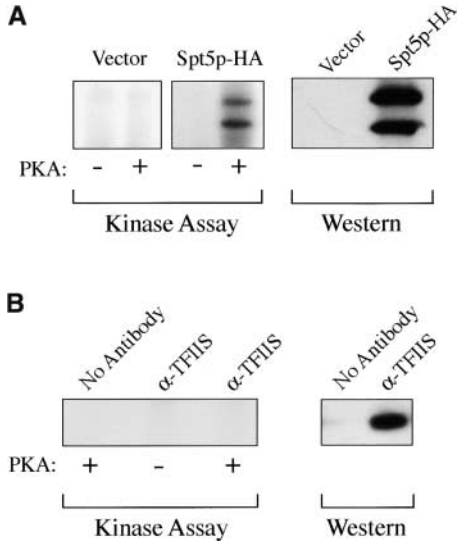


FIGURE 8.—Spt5p was phosphorylated by PKA *in vitro*. (A) Examination of Spt5p phosphorylation by PKA. Wild-type cells (PHY1025) carrying either a plasmid encoding an HA epitope-tagged version of Spt5p (pPHY1720) or a control plasmid (pPHY1723) were cultured as described in MATERIALS AND METHODS. Cell extracts were prepared and HA-Spt5p was precipitated with a monoclonal antibody specific for the HA epitope that was conjugated to agarose beads. The immunoprecipitated material was split into three aliquots and then analyzed either with a PKA *in vitro* phosphorylation assay or by Western immunoblotting. For the PKA phosphorylation assays, 1 μCi of [γ-^{32}P]ATP and either 0 or 10 unit of bovine PKA (Sigma) were added to two of the above three aliquots. These reactions were incubated for 30 min at 25° and the reaction products were then eluted from the agarose beads and separated on a 7.5% SDS-polyacrylamide gel. Following electrophoresis, the gel was fixed, dried, and subjected to autoradiography. For the Western immunoblot analysis, the proteins in the third aliquot of the immunoprecipitated material were eluted from the agarose beads and separated on a 7.5% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and the HA-Spt5p present was detected by Western immunoblotting with an antibody specific for the HA epitope. (B) Examination of Ppr2p phosphorylation by PKA. Cell lysates were prepared from wild-type cells (PHY1942) as described in MATERIALS AND METHODS. Ppr2p was precipitated from these lysates with a polyclonal antiserum specific for the TFIIS elongation factor. The precipitated protein was then analyzed in a PKA kinase assay and by Western immunoblotting essentially as described for Spt5p in A.

DISCUSSION

Previous studies have suggested that the Ras/PKA signaling pathway in *S. cerevisiae* regulates RNA pol II transcription and that this control is exerted, at least in part, at the level of the Rpb1p CTD (HERMAN 2002; HOWARD *et al.* 2002). The work presented here extends these observations and suggests that this regulation might be acting at the level of RNA pol II transcript elongation. In particular, this study suggests that the Ras/PKA pathway is controlling the activity of the Spt4p/Spt5p complex, the *S. cerevisiae* equivalent of the mammalian DSIF elongation factor. This assertion is supported by multiple lines of evidence. First, *RAS2*^{val19} and *rpb1-104* mutants are sensitive to drugs, like 6AU and MPA, that inhibit the growth of mutants defective for

RNA pol II transcript elongation. Second, elevated levels of Ras/PKA signaling activity inhibited the growth of a subset of mutants defective for RNA pol II elongation; this subset included the *spt4* and *spt5* mutants. Third, the *RAS2*^{val19}, *rpb1-104*, *spt4*, and *spt5* mutants all exhibited a similar transcriptional response to the drugs 6AU and MPA. Fourth, *spt5* mutants, like strains containing either the *RAS2*^{val19} or *rpb1-104* alleles, were unable to enter into a normal stationary phase in response to nutrient deprivation. Finally, Spt5p was an efficient *in vitro* substrate for PKA. Altogether, we feel that these observations are most consistent with a model proposing that the Ras/PKA pathway regulates RNA pol II transcript elongation by directly targeting Spt5p within the Spt4p/Spt5p elongation factor complex.

Other models could be invoked to explain some of the experimental observations made in this study. However, we feel these alternatives are less able to account for the full complement of genetic and biochemical data presented here. For example, one of our initial concerns was that the drugs 6AU and MPA affected the intracellular levels of GTP, a key regulator of the Ras/PKA signaling pathway (Broach 1991; Herman 2002). In fact, one study has gone so far as to suggest that the activity of the *S. cerevisiae* Ras/PKA pathway is directly controlled by the intracellular levels of GTP (Haney and Broach 1994). Therefore, it was formally possible that the 6AU- and MPA-associated growth defects observed in the Ras pathway mutants were a direct consequence of the altered GTP levels. However, this model would predict that these drugs would preferentially inhibit the growth of mutants that have decreased levels of Ras signaling activity and we observed the opposite result in this study. A second possibility was that the presence of the *RAS2*^{val19} allele might greatly alter the transcriptional properties of the yeast cell and thus render the cells more sensitive to changes in nucleotide levels. However, a recent whole-genome analysis of gene expression in *RAS2*^{val19} mutants has failed to identify the dramatic changes in RNA pol II transcription implicit in this model (our unpublished data; see also Howard *et al.* 2002). These microarray experiments found that $<10\%$ of the yeast transcriptome was altered by more than twofold in a *RAS2*^{val19} mutant, relative to the wild-type controls. Ultimately, it will be interesting to see if the genes affected by elevated levels of Ras signaling activity are similarly affected by *spt5* mutations. Finally, the data are inconsistent with any model suggesting that the drug sensitivity of the *RAS2*^{val19} mutant was due to diminished levels of GTP, or other nucleotides, in this mutant. In such a model, we would have expected that all of the mutants that were sensitive to 6AU (and MPA) would have been similarly sensitive to the presence of the *RAS2*^{val19} allele. This was not the case as we observed a clear specificity in the genetic interactions with the *RAS2*^{val19} mutation. Thus, we feel that the data presented here are best explained by the Ras/PKA pathway having a role in the regulation of RNA pol II elongation.

The *in vitro* phosphorylation by PKA identifies Spt5p as a potential target of the Ras/PKA pathway that could be responsible for the observations made in this study. However, further work will be necessary to confirm that Spt5p is indeed phosphorylated by PKA *in vivo*. Unfortunately, this analysis will be complicated by the fact that the yeast Spt5p, like its mammalian counterpart, is heavily phosphorylated *in vivo*. To circumvent this problem, the immediate strategy will be to identify the Spt5p sites that are phosphorylated by PKA *in vitro* and to then test whether these sites are responsible for the sensitivity of the *RAS2*^{val19} mutant to 6AU, MPA, and decreased Spt4p/Spt5p activity. This analysis should reveal the physiological relevance of the Spt5p phosphorylation observed in this study.

A separate, but equally important, question concerns the potential regulatory role that might be played by the Ras/PKA pathway. Previous studies have suggested that the Spt4p/Spt5p complex has a dual role during RNA pol II elongation. In the early stages of the elongation process, this complex is thought to inhibit the transition of the polymerase to an elongation-competent form (Wada *et al.* 1998a,b; Yamaguchi *et al.* 1999). This activity might serve as a type of checkpoint control to ensure that the polymerase does not begin elongating the mRNA transcript until the appropriate polymerase complex is assembled. At later stages, the Spt4p/Spt5p complex appears to stimulate the processivity of the elongating polymerase (Wada *et al.* 1998a; Ping and Rana 2001). Although our data do not directly address this issue, we presently favor the possibility that the Ras/PKA pathway would regulate the former activity. One reason for this preference is that it seems counterintuitive that a pathway providing a signal for growth would directly inhibit RNA pol II transcript elongation. Instead, Ras/PKA activity might normally function to stimulate the switch between initiation and elongation modes of the polymerase. In mutants with high Ras activity, this switch might occur prematurely and thus result in specific defects in RNA pol II elongation.

We have suggested previously that the Ras/PKA pathway might be regulating gene expression by directly targeting proteins that are physically associated with the RNA pol II enzyme (Chang *et al.* 2001; Herman 2002). This type of a control mechanism differs from the better-characterized mode employed by signaling pathways that are responding to changes in the extracellular environment. In general, these signaling pathways target regulatory proteins that are bound to the enhancer or upstream activating sequence elements that are typically upstream of the gene of interest. By directly targeting proteins associated with the polymerase, these signaling pathways could potentially influence gene expression from a large number of promoters with a single regulatory event. Although a definitive example of this type

of a control mechanism has not yet been described, several reports have suggested that proteins in the basal RNA pol II machinery may be regulated in this manner (Jiang *et al.* 1998; Kuchin *et al.* 2000; Zhang and Emmons 2000; Chang *et al.* 2001). To date, all of these potential targets have been proteins important for RNA pol II transcription initiation. Here, we present data suggesting that the Ras/PKA pathway may be directly targeting a component of the elongating polymerase complex and that this regulation is important for the normal control of yeast cell growth. These data therefore point out the interesting possibility that signaling pathways might also directly influence the elongation step of RNA pol II transcription.

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