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; Manciniak 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-ribofuransylbenzimidazole (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. 1998b). 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 transcription 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; Shefls 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; Hirsch 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 nitrogen 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<sup>val19</sup> allele cloned into pRS415, pRS416, and pRS414, respectively. The pRS plasmids were described previously (Sikorski and Hieter 1989). The LEU2-marked MET3-RAS2<sup>val19</sup> plasmid, pPHY795, was constructed as described (Howard et al. 2002). The high-copy PDE2 plasmid, pPHY1299, was constructed by subcloning a 2.4-kb BamHI-HindIII fragment that contains PDE2 into pRS425. The high-copy IMD2 plasmid, pPHY1410, was constructed by subcloning the XhoI-NcoI fragment containing IMD2 from pPHY1401 into pRS425; pPHY1401 was provided by Daniel Reines and is a pRS426-based plasmid that contains IMD2. The pGHis1 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<sup>val19</sup> allele, strains were transformed with either pPHY453 or pPHY795 (MET3-RAS2<sup>val19</sup>). The pPHY795 transformants were recovered on media containing 500 μM methionine and then grown on medium lacking methionine to induce expression of RAS2<sup>val19</sup>. The rpb1-I104 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 rpb1-I18 allele. This strain was trans-
formed with a LEU2-marked rpb1-104 plasmid, pHY854, 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 polyacrylamide gel. The gel was fixed with a 10% trichloroacetic acid/10% acetic acid/50% methanol solution, dried, and a hot phenol extraction method described previously (Costa and Arndt, 1998). The RNA was precipitated to an antibody specific for the HA epitope (Roche, Indianapolis). The beads were washed with TBS and resuspended in kinase reaction buffer (10 mM Tris-HCl, pH 7.4, 140 mM NaCl). Cell lysates were incubated overnight at 4°C 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 MgCl₂, 4.5 mM dithiothreitol, 5 mM NaF, 50 mM KPi, pH 7.15) containing 1 μCi [γ-³²P]ATP (Perkin-Elmer, Norwalk, CT) and 10 units of bovine PKA (Sigma). The reactions were incubated for 30 min at 25°C, 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 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. PHY942 cells were grown to midlog phase in YPAD medium and cell lysates were prepared from eight OD₆₀₀ unit equivalents of cells. The lysates were incubated overnight at 4°C 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 1

**Yeast strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Alias</th>
<th>Source/Reference</th>
</tr>
</thead>
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<tr>
<td>AHJ2</td>
<td>MATα leu2Δ1 his3Δ200 ura3-52 ctkΔ1::HIS3</td>
<td>PHY1872</td>
<td>Lee and Greenleaf (1991)</td>
</tr>
<tr>
<td>DY5393</td>
<td>MATα spo16 (G132D) ade2 can1 his3 leu2 trp1 ura3</td>
<td>PHY3599</td>
<td>David Stillman</td>
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<td>FY267</td>
<td>MATα his4-912 lys2-1286 leu2Δ1</td>
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</tr>
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<td>FY1646</td>
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<td>Hartzog et al. (1998)</td>
</tr>
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<td>FY1645</td>
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<td>FY1667</td>
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<tr>
<td>KY499</td>
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<td>GHY283</td>
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<td>PHY1942</td>
<td>MATα his3Δ200 leu2-3,112 lys2-801 pbl1::hisG prc1::HIS3 pep4::LEU2</td>
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<td>Lee and Greenleaf (1991)</td>
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<td>PHY2848</td>
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<td>MATα his3Δ200 ura3-52 rpb1Δ187::HIS3 p(rpb1-104/LEU2)</td>
<td>PHY8416</td>
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</tr>
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<td>PHY2914</td>
<td>MATα his3Δ200 ura3-52 rpb1Δ187::HIS3 p(RPB1/LEU2)</td>
<td>PHY8416</td>
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</tr>
<tr>
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<td>Lee and Greenleaf (1991)</td>
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</table>
TABLE 2

RAS2<sup>ald19</sup> mutants were very sensitive to drugs that inhibit the growth of mutants defective for RNA polymerase II transcript elongation

<table>
<thead>
<tr>
<th></th>
<th>No drug</th>
<th>6AU</th>
<th>MPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>RAS2&lt;sup&gt;ald19&lt;/sup&gt;</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>&lt;i&gt;spt4&lt;/i&gt;Δ</td>
<td>++</td>
<td>–</td>
<td>–</td>
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<tr>
<td>&lt;i&gt;spt5-194&lt;/i&gt;</td>
<td>++</td>
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<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>&lt;i&gt;ppr2Δ&lt;/i&gt;</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ras2-23</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

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

RESULTS

RAS2<sup>ald19</sup> 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<sup>ald19</sup>, were found to be as sensitive to both 6AU and MPA as any of the previously described elongation mutants (Table 2). The RAS2<sup>ald19</sup> 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<sup>ald19</sup>, <i>ppr2Δ</i>, and <i>spt4Δ</i>, are shown in Figure 1. In contrast to these results with RAS2<sup>ald19</sup>, 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 ras1Δ 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 <i>PDE2</i> gene was introduced into a RAS2<sup>ald19</sup> mutant. <i>PDE2</i> encodes a high-affinity cAMP phosphodiesterase that, when overproduced, results in lowered levels of PKA activity (Sass et al. 1986). We found that this <i>PDE2</i> plasmid suppressed the 6AU sensitivity of RAS2<sup>ald19</sup> 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; Desmouelles 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<sup>ald19</sup> 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<sup>ald19</sup> 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 (sct22). For most of these experiments, the RAS2<sup>ald19</sup> coding sequences were fused to the inducible promoter from the <i>MET3</i> gene (Howard et al. 2001). This promoter is active in cells grown in media lacking methionine and is repressed by the presence of...
methionine in the growth medium (Cherest et al. 1987; Mountain et al. 1991; Howard et al. 2002).

The expression of RAS2\textsuperscript{val19} resulted in a strong synthetic growth defect in the spt4, spt5, and spt6 mutants (Figure 2). In addition, RAS2\textsuperscript{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\textsuperscript{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\textsuperscript{val19} (Howard et al. 2002).

Interestingly, not all mutations thought to compromise transcript elongation were affected by the presence of RAS2\textsuperscript{val19} (Figure 2). For example, RAS2\textsuperscript{val19} was not synthetically lethal with mutations that affect the activity of the elongation factor, TFIIIS. These mutations included a disruption of PPR2, the gene encoding the S. cerevisiae TFIIIS, and rpo21-18, an allele of RPB1 that lowers TFIIIS 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\textsuperscript{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 ppr2Δ and rpo21-18 (Shaw and Reines 2000; Shaw et al. 2001). Finally, several spt16 mutants,

**Figure 1.—** RAS2\textsuperscript{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 \( \mu g/\text{ml} \) 6AU, or 30 \( \mu g/\text{ml} \) MPA and incubated for 3 days at 30°C. The strains analyzed were wild type (FY267), RAS2\textsuperscript{val19} (FY267 carrying the RAS2\textsuperscript{val19} plasmid, pPHY453), spt6Δ (FY1646), and ppr2Δ (GHY285). All strains, except the RAS2\textsuperscript{val19} mutant, were carrying the control vector, pRS415.

**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°C. The strains all carried a plasmid, pPHY795, that contains the MET3-RAS2\textsuperscript{val19} allele and were grown under conditions in which the MET3 promoter was either repressed (RAS2; 500 \( \mu g/\text{ml} \) methionine) or active (RAS2\textsuperscript{val19}; 0 \( \mu g/\text{ml} \) methionine). The strains analyzed were wild type (FY267), spt6Δ (FY1646), spt6-14 (FY1555), spt6-50 (FY1667), rtf1Δ (FY1559), rpb1-221 (GHY190), rpb1-244 (GHY149), and ppr2Δ (GHY285). (B) The relative growth rate of the indicated strains containing either a wild-type (RAS2) or hyperactive (RAS2\textsuperscript{val19}) allele of the RAS2 locus was assessed after 3 days of growth on minimal medium at 30°C. For the RAS2\textsuperscript{val19} experiments, strains were carrying the MET3-RAS2\textsuperscript{val19} allele and were grown in medium lacking methionine to allow for the expression of RAS2\textsuperscript{val19}. 

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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.

These genetic interactions with Ras2<sup>val19</sup> 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<sup>val19</sup> 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<sup>val19</sup> and spt mutants were not defective for the induction of IMD2.** Previous work has suggested that the 6AU- and MPA-sensitive phenotypes of ppr2Δ, 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 ppr2Δ, rpo21-18, and rpb2-10 mutants (Shaw et al. 2001). We tested whether Ras2<sup>val19</sup> and the drug-sensitive spt4Δ and spt5-194 mutants were similarly defective in inducing IMD2 transcription following an exposure to 6AU or MPA. Interestingly, we found that the Ras2<sup>val19</sup> 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<sup>val19</sup> 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 ppr2Δ and rpo21-18 mutants (Figure 4) and to a lesser degree in rtf1 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<sup>val19</sup> and spt mutants induced IMD2 expression to an almost wild-type level (Figure 5). This differed dramatically from the MPA effects on the ppr2Δ and rpo21-18 mutants. In these latter mutants, treatment with MPA resulted in a slight decrease in the levels of IMD2 mRNA.
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 ppr2Δ, rpb2-10, rpo21-18, and rtf1Δ mutants fall into the former class, whereas spt4Δ and spt5-194 mutants can be placed into the latter. Interestingly, these molecular data established a functional link between the RAS2α19 and spt mutants and were generally consistent with the genetic interactions observed above. In particular, RAS2α19 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 ppr2Δ, rpo21-18, or rpb2-10 mutants. This correlation was not absolute, however, as the rtf1Δ mutant exhibited a synthetic growth defect with RAS2α19, 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 spt4Δ 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, RAS2α19, 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 RAS2α19 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 RAS2α19, 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 Y7-S2-T4-S5-P6-S7 (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 RAS2α19 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 RAS2α19, 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 RAS2α19 cells. The Ras/PKA pathway plays a central role in regulating growth in response to changes in nutrient availability. Cells with the RAS2α19 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 \textit{RAS2}^{val19} and \textit{spt} mutants. (A) \textit{RAS2}^{val19} and \textit{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 \textit{RAS2}^{val19} genetic interactions were characterized either in this study or in previous work from our lab (Howard et al. 2002), and the \textit{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°C on YM glucose medium that contained no drug, 125 \( \mu \)g/ml 6AU, or 30 \( \mu \)g/ml MPA. The strains analyzed were wild type (PHY2195), \textit{RAS2}^{val19} (PHY2194), and \textit{rpb1-104} (PHY2193). (C) The \textit{rpb1-104} CTD truncation mutant exhibited wild-type levels of \textit{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°C and were incubated for an additional 3 hr at 30°C. Total RNA was prepared from each of the three aliquots, and the levels of \textit{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 \textit{IMD2} mRNA in the drug-containing cultures to that found in the no drug control. In all cases, the relative amount of \textit{IMD2} mRNA was normalized to that of the loading control, \textit{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 \textit{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 \textit{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, \textit{rpb1-104} (Howard et al. 2002). The \textit{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 \textit{spt5-194} cells, like both the \textit{RAS2}^{val19} and \textit{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.—\textit{RAS2}^{val19} and \textit{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°C. Fivefold serial dilutions of these cultures were then spotted to a solid medium and incubated for 4 days at 30°C. 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), \textit{RAS2}^{val19} (FY267 with pPHY453), \textit{spt5}Δ (FY1646), \textit{spt5-194} (GHY379), and \textit{ppr2}Δ (GHY285). All of the strains except the \textit{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 ppr2Δ,
that were defective for the induction of IMD2 expres-
sion. The ppr2Δ stationary phase cultures contained es-
sentially the same number of survivors after 9 days of
growth as the wild-type strain (Figure 7). In addition,
the spt4Δ mutant did not exhibit defects in either cell
survival or glycogen accumulation during the stationary
phase of growth (Figure 7 and data not shown). There-
fore, the spt5 mutants were unique among the elonga-
tion mutants with respect to these stationary phase de-
fects.

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 RAS2Δ mutant. Second,
spt5 mutants were the only elongation-
defective mutants that exhibited stationary phase-spe-
cific phenotypes similar to those observed with RAS2Δ.
Third, RAS2Δ 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 respon-
sible 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 experi-
ments, Spt5p, and other potential targets, were immu-
noprecipitated from cell extracts and then incubated
with [γ-32P]ATP in the presence, or absence, of the PKA
enzyme. Spt5p was found to be efficiently phosphory-
lated 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 phos-
phorylation of Spt5p could have been due to a PKA-activated
protein kinase that was present in the immunoprecipi-
tates, the intensity of the phosphorylation signal was
more consistent with Spt5p being a direct target for
PKA. Thus, these biochemical data support the proposi-
tion that the Spt4p/Spt5p complex is a direct target of the
Ras/PKA signaling pathway.

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 mam-
alian DSIF elongation factor. This assertion is sup-
ported by multiple lines of evidence. First, RAS2Δ 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 \textit{RAS2}^{val19}, \textit{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 \textit{RAS2}^{val19} or \textit{rpb1-104} alleles, were unable to enter into a normal stationary phase in response to nutrient deprivation. Finally, Spt5p was an efficient \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{RAS2}^{val19} allele. This was not the case as we observed a clear specificity in the genetic interactions with the \textit{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 \textit{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 \textit{in vivo}. Unfortunately, this analysis will be complicated by the fact that the yeast Spt5p, like its mammalian counterpart, is heavily phosphorylated \textit{in vivo}. To circumvent this problem, the immediate strategy will be to identify the Spt5p sites that are phosphorylated by PKA \textit{in vitro} and to then test whether these sites are responsible for the sensitivity of the \textit{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 stabilize 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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