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Mutations in the *Saccharomyces cerevisiae RPB1* Gene Conferring Hypersensitivity to 6-Azauracil

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ABSTRACT

RNA polymerase II (RNAPII) in eukaryotic cells drives transcription of most messenger RNAs. RNAPII core enzyme is composed of 12 polypeptides where Rpb1 is the largest subunit. To further understand the mechanisms of RNAPII transcription, we isolated and characterized novel point mutants of *RPB1* that are sensitive to the nucleotide-depleting drug 6-azauracil (6AU). In this work we reisolated the *rpo21-24/rpb1-E1230K* allele, which reduces the interaction of RNAPII–TFIIS, and identified five new point mutations in *RPB1* that cause hypersensitivity to 6AU. The novel mutants affect highly conserved residues of Rpb1 and have differential genetic and biochemical effects. Three of the mutations affect the "lid" and "rudder," two small loops suggested by structural studies to play a central role in the separation of the RNA–DNA hybrids. Most interestingly, two mutations affecting the catalytic center (*rpb1-N488D*) and the homology box G (*rpb1-E1103G*) have strong opposite effects on the intrinsic *in vitro* polymerization rate of RNAPII. Moreover, the synthetic interactions of these mutants with *soh1*, *spt4*, and *dst1* suggest differential *in vivo* effects.

RNA polymerase II (RNAPII) is composed of 12 polypeptides with a high degree of structural conservation from yeast to humans (Hahn 2004). In *Saccharomyces cerevisiae*, Rpb1 and Rpb2 form the central part of RNAPII and share an ample contact surface. The interaction between both subunits shapes several domains of the enzyme, such as the active center, which is formed by the "active site" and the "hybrid-binding" regions of Rpb1 and Rpb2, respectively (Cramer *et al.* 2001). The functional complexity of RNAPII is reflected in its structure, revealing the existence of a large number of regions with very specific functions. The core polymerase requires the association of a number of initiation factors for promoter recognition and initiation of RNA synthesis. Once in elongation mode, the crosstalk between RNAPII and a number of associated factors assures a proper mRNA synthesis (Sims *et al.* 2004). To gain further insight into the process of transcription, we have isolated mutants of *RPO21/RPB1*, encoding the largest subunit of the RNAPII in yeast, obtained by a random mutagenesis. The mutants were selected by their sensitivity to 6-azauracil (6AU), a drug that decreases GTP and UTP pools (Exinger and Lacroute 1992). We reasoned that leaky mutants of RNAPII might be susceptible to imbalances in the intracellular nucleotide pools at steps in initiation, elongation, or termination.

Sensitivity to 6AU is a well-documented phenotype associated with transcription-elongation mutants. For example, the $6AU^S$ mutant *rpb2-10* (P1018S) is intrinsically arrest prone and has a slower polymerization rate (Powell and Reines 1996; Mason and Struhl 2005). Further, yeast knockouts of the genes *DST1/PPR2* and *SPT4*, encoding the transcription elongation factors TFIIS (Fish and Kane 2002) and Spt4 (Hartzog *et al.* 1998), are highly sensitive to 6AU. As expected, the *rpb1-E1230K* (*rpo21-24*) point mutant, which decreases the binding of TFIIS to RNAPII (Archambault *et al.* 1992; Wu *et al.* 1996), is also $6AU^S$. Biochemical analyses have shown that TFIIS stimulates transcription elongation, increases the fidelity of incorporation of ribonucleotides, and is essential for the reactivation of arrested RNAPII *in vitro* (Fish and Kane 2002). Apart from the well-characterized role of TFIIS in elongation *in vitro*, reports from different laboratories show that TFIIS is also involved in transcription initiation (Davie and Kane 2000; Malagon *et al.* 2004; Adelman *et al.* 2005; Prather *et al.* 2005). Thus, it is possible that some of the sensitivity to 6AU in a *dst1* knockout is due to defects in initiation caused by lack of TFIIS, and it might be argued that some mutants in RNAPII that are sensitive to 6AU would be arrest prone or compromised in initiation of transcription. Although no single *rpb1* or *rpb2* $6AU^S$ mutant is affected in initiation, the *rpb2-101* (G369S) mutation is $6AU^S$ and has an altered transcription initiation in the presence of a mutant TFIIB initiation factor (Chen and Hampsey 2004).

We report here the isolation and characterization of several novel $6AU^S$ alleles of *RPB1*. These include alterations of conserved domains of RNAPII near the

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TABLE 1

Yeast strains

Strain	Relevant genotype[a]	
GRY3019	*PtetRPB1*[b]	*RPB3*
GRY3020	*RPB1*	*RPB3*
GRY3021	*rpb1-C67Y*	*RPB3*
GRY3022	*rpb1-C70Y*	*RPB3*
GRY3023	*rpb1-H80Y*	*RPB3*
GRY3024	*rpb1-D260N*	*RPB3*
GRY3025	*rpb1-D261N*	*RPB3*
GRY3026	*rpb1-R320C*	*RPB3*
GRY3027	*rpb1-N488D*	*RPB3*
GRY3028	*rpb1-E1103G*	*RPB3*
GRY3029	*rpb1-E1230K*	*RPB3*
GRY3030	*PtetRPB1*	*RPB3::6His*
GRY3031	*RPB1*	*RPB3::6His*
GRY3036	*rpb1-D261N*	*RPB3::6His*
GRY3037	*rpb1-R320C*	*RPB3::6His*
GRY3038	*rpb1-N488D*	*RPB3::6His*
GRY3039	*rpb1-E1103G*	*RPB3::6His*
GRY3040	*rpb1-E1230K*	*RPB3::6His*
GRY3100	*PtetRPB1*	*RPB3 dst1Δ*
GRY3101	*PtetRPB1*	*RPB3 dst1Δ*

[a] All strains are *MAT***a** with the exception of *GRY3100* (*MAT*α).
[b] *kanRPtetO7-TATA-RPB1.*

TABLE 2

Oligonucleotides

Name	Sequence[a]
RPB1-C:	GAACATCCGATTCGTTCATTAACTGAAATC
RPB1-1f:	CCAAATTTCAAAAAAATTTTACC
RPB1-2f:	AAAGTATGTGAGTGTGTCTG
RPB1-2r:	CAGACACACTCACATACTTT
RPB1-3f:	TGATTTTAACATGCCTTCCT
RPB1-4f:	TCTATTGCCAAGACTTTAAC
RPB1-4r:	GTTAAAGTCTTGGCAATAGA
RPB1-5f:	CTTTCTCAATTATGTGCTGT
RPB1-6f:	CGAGAGAAAAGGGACCTCAA
RPB1-7f:	GCAATCTGTTGAAGGTAAAC
RPB1-8f:	TATTGAATACAGACCATACC
RPB1-8r:	GGTATGGTCTGTATTCAATA
RPB1-11f:	GAACCTGAATGGGTGTTGGA
RPB1-11r:	TCCAACACCCATTCAGGTTC
RPB1-12f:	GGTACCGGTGCATTTGATGT
RPB1-12r:	ACATCAAATGCACCGGTACC
RPB1-14f:	CCTTCTTACTCCCCAACATC
RPB1-15r:	TTCCCTATCCCTACCATAATGC
RPB1-D:	TAACATTACCAGATCACGGAAATACGCAAG
IMD2f:	GTGGTATGTTGGCCGGTACTACCG
IMD2r:	TCAGTTATGTAAACGCTTTTCGTA
SED1f:	ACTTCAGCTTTCCCACCAACT
SED1r:	GACAACGGAATGAGAAGAAGCA
ACT1f:	CTGCTGAAAGAGAAATTGTCCG
ACT1r:	CTTGTGGTGAACGATAGATGGA
RND25-1f:	TTGGAGAGGGCAACTTTGG
RDN25-1r:	CAGGATCGGTCGATTGTGC
PRB1f:	TAAGAGGCTTTTTGAACACTGCATTGCACC
PRB1r:	ACTTCAAAAGAACCCTCGGTATAATTCCTG
RPB3P5:	TTTTCACCGGTGACCCAGAGCAA
HISNEL:	AATTCGAGTGGTGGTGATGGTGGTGTGAACC
NDS45G:	GGTATAGGATACTTACAGCCATCGAGAGG GACAAGGCGAAAAGAG
TDS45G:	CTCTTTTCGCCTTGTCCCTCTCGATGGCTGT AAGTATCCTATACC
rna9:	AUCGAGAGG

[a] Oligonucleotide sequences are presented 5′–3′.

active site, the point where the DNA–RNA hybrid separates (the lid and rudder domains), and the region where the template and nontemplate strands of the DNA downstream of the active site separate. In addition, we reisolated the *rpb1-E1230K* allele that blocks TFIIS binding (Wu *et al.* 1996). Biochemical characterization of these 6AU[S] alleles demonstrates that they have different consequences on elongation. Similarly, genetic characterization shows that they have different dependency on other transcription factors. Finally, these mutations have different consequences for the expression of some genes normally involved in response to 6AU. Combined, these results suggest that 6AU sensitivity can be caused by defects in several different aspects of transcription and that 6AU[S] *rpb1* mutants can be obtained that reveal these different functions.

MATERIALS AND METHODS

Media, yeast manipulations, strains, plasmids, and oligonucleotides: Media, growth conditions, and yeast manipulations were as previously described (Malagon *et al.* 2004). Sensitivity to 6AU was scored on AA-Ura + 6AU plates by replica plating (100 μg/ml 6AU) or serial dilutions (10 μg/ml 6AU). All strains used are direct derivatives or closely related to the BY series of the yeast knockout collection into which we introduced different alleles of *RPB1* (see Table 1). Strains GRY3019–GRY3029 are all *his3Δ leu2Δ lys2Δ met15Δ trp1Δ::hisG URA::CMV-tTA*. GRY3100 and GRY3101 are *his3Δ leu2Δ met15Δ trp1Δ::hisG URA::CMV-tTA dst1Δ::natMX4*. Strains GRY3030–GRY3040 are *his3 leu2 lys2Δ met15Δ trp1 can1 pep4::HIS3 prb1Δ1.6R RPB3::6xHis URA::CMV-tTA* and are related to the BY yeast knockout collection and to BJ5464 *MAT*α *can1 his3Δ200 leu2Δ1 trp1 ura3-52 pep4::HIS3 prb1Δ1.6R* GAL+ (American Type Culture Collection Yeast Genetic Stock Center). The *PtetRPB1* allele and the tTA transactivator were introduced by crosses with the strain YSC1180-7428981 (Open-Biosystems; Mnaimneh *et al.* 2004). All yeast strain relevant genotypes are described in Table 1. All oligonucleotides used are shown in Table 2. Plasmid pL-RPB1 is a *LEU2*-based centromeric plasmid containing the *RPB1* gene from position −595 to +5754 relative to the start of the open reading frame. The plasmids containing the *rpb1* mutations were named pL-rpb1-*x* (*x* represents the specific allele).

***RPB1* mutagenesis and sequencing of the mutations:** Plasmid pL-RPB1 was mutagenized using the mutator XL1-Red competent cells kit, following the supplier's recommendations (Stratagene, La Jolla, CA). The genotype of the *Escherichia coli* strain XL1-red is *endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10* (Tet[r]). Sequencing a region covering the *RPB1* gene from positions −509 to +5641 identified the mutations.

Integration of *RPB1* mutants: The wild-type and mutant alleles of *RPB1* were introduced by homologous recombination.

For that purpose, strains GRY3019 and GRY3030 were transformed with linear fragments from the pL-RPB1 series obtained by digestions with *Ava*I (located in the *RPB1* promoter proximal polylinker) and *Eag*I, *Xba*I, *Kpn*I, or *Hin*dIII, depending on the location of the mutation of interest. Recombinants replace the G418^{r}Ptet with the natural *RPB1* promoter and were therefore selected by growth in doxycycline-containing media and subsequently screened for sensitivity to G418. The proper integrations were then confirmed by sequencing.

Northern analysis: Northern analysis was done basically as described (Shaw and Reines 2000). Briefly, total RNA was extracted with acid phenol from cells growing exponentially in AA-Ura media. The final concentration of 6AU was 75 μg/ml, and samples were taken at times 0, 30, and 120 min. RNA transfer and hybridization were done using the NorthernMax^R kit (Ambion, Austin, TX). Radioactive labeled probes for *IMD2*, *SED1*, *ACT1*, and *RDN25-1* were obtained from PCR fragments, using the corresponding primers (Table 2). The *SSM1* probe was obtained from a 0.5-kb *Bsm*I–*Pvu*I internal fragment of the gene from plasmid YEplac181-SSM1 (F. Malagon, unpublished data).

Transcription *in vitro*: RNAPII purification and transcription complex reconstitution were done as previously described (Kireeva *et al.* 2003). Briefly, RNAPII was purified from yeast cell extracts by attachment of hexahistidine-tagged Rpb3 to Ni^{2+}-NTA agarose beads. The 5′ radioactively labeled RNA (rna9) and the template DNA strand (TDS45G) oligonucleotides were incubated with the immobilized RNAPII. The nontemplate DNA strand (NDS45G) was subsequently added. Elongation of the RNA was allowed to proceed by adding NTPs at a final concentration of 10 μm. The products were resolved in 20% denaturing polyacrylamide gels.

Sequence alignment analysis: Sequence alignment was done using the Clustal W program (Thompson *et al.* 1994).

Crystal structure visualization: RNAPII transcription complex PDB:1SFO (Westover *et al.* 2004) visualization and localization of specific residues were done using Protein Explorer (www.proteinexplorer.org/) (Martz 2002).

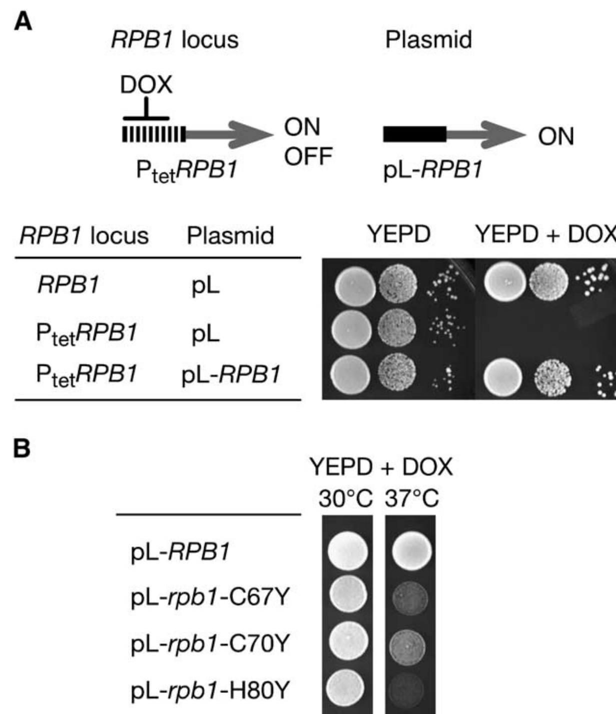


Figure 1.—Isolation of plasmid-borne *rpb1* mutants. (A) Schematic representation and *in vivo* validation of the strategy used to isolate plasmid-borne *rpb1* recessive mutants. Shown are serial 50-fold dilutions, containing different combinations of *RPB1* alleles as indicated, plated in rich media without or with doxycycline. The striped and solid boxes represent a doxycycline repressible tet promoter and the *RPB1* natural promoter, respectively. The shaded arrow represents the *RPB1* open reading frame. YEPD, rich media; DOX, doxycycline. (B) The growth phenotype of plasmid-borne thermosensitive *rpb1* mutants isolated using the strategy depicted in A. YEPD, rich media; DOX, doxycycline.

RESULTS

Because the largest subunit of RNAPII (encoded by *RPB1*) is essential and we wanted to be able to identify recessive mutations, we developed a screen for *rpb1* mutants on the basis of a strain (GRY3019) that contains an *RPB1* allele (*PtetRPB1*) that can be turned off by addition of doxycycline to the media (Mnaimneh *et al.* 2004). GRY3019 does not survive in the presence of doxycycline, but can be rescued by a low-copy plasmid, pL-RPB1, carrying the *RPB1* gene with its own promoter (Figure 1A). We introduced a mutagenized pool of pL-RPB1 into GRY3019 and then screened for novel phenotypes that were revealed when the chromosomal *RPB1* allele was turned off.

Isolation of temperature-sensitive *rpb1* mutants: To validate the mutant isolation strategy, we first looked for mutants with a robust growth phenotype in rich media at 30° but that were unable to grow at 37°. We chose this phenotype because of the ease of scoring it and the fact that a number of temperature-sensitive (TS) *rpb1* mutants have already been described. Of 32,000 independent clones analyzed, 11 showed the TS phenotype. Due to the relatively long size of the *RPB1* ORF, 5.2 kb, a high level of comutations could potentially complicate the identification of the relevant mutations. The sequencing of the candidate clones revealed five single, five double, and one triple mutant. Therefore, this mutagenesis level is sufficient to obtain the desired clones without the inconvenience of having too many secondary mutations. In agreement with the lethal phenotype of deletions of the carboxyl-terminal domain of *rpb1* (Nonet *et al.* 1987), none of the mutations were out-of-frame deletions or insertions. The sequencing data uncovered unambiguously the temperature-sensitive alleles *rpb1-C67Y*, *rpb1-C70Y*, and *rpb1-H80Y*, isolated from two, five, and three independent clones, respectively (Figure 1B). An additional mutation, causing the residue change C103Y, was also isolated but was not further analyzed due to the presence of secondary mutations. To avoid possible artifacts due to the plasmid-borne expression of the *rpb1* mutants or low-level expression of the *PtetRPB1* allele, the mutations were integrated in the chromosomal *RPB1* locus and tested for growth at 30° and 37° (Figure 2). It is interesting that

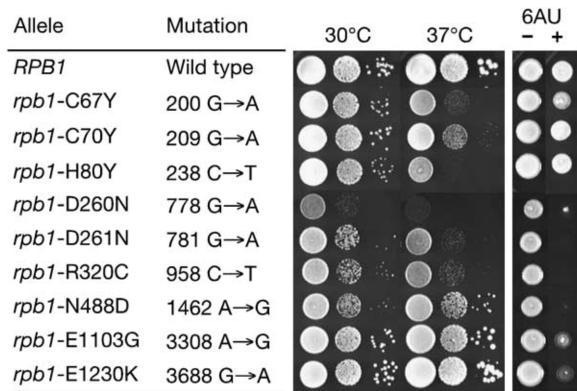


FIGURE 2.—Isolation and identification of *rpb1* mutants sensitive to 6AU. Shown are phenotypes of mutations in *RPB1* causing sensitivity to 6AU, isolated using the strategy shown in Figure 1 or a related strategy (see text), after integration in the chromosome. Integrated versions of the *rpb1* thermosensitive mutants are also shown. The allele names indicate the amino acid change in Rpb1. Mutation positions are relative to the first nucleotide in the open reading frame of *RPB1* and the changes shown correspond to the nontranscribed strand. All the mutants contain a single-base substitution in *RPB1*. 6AU, 6-azauracil.

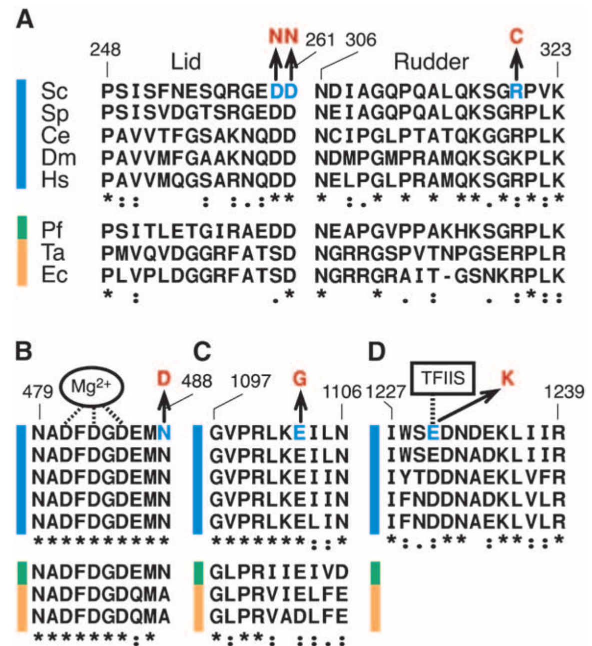


FIGURE 3.—Evolutionary conservation of Rpb1-mutated residues. Sequence alignments covering the entire lid and rudder (A), regions in the active site (B), homology box G (C), and TFIIS binding (D) are shown. The numbers refer to the codon of the *S. cerevisiae* open reading frame. The *S. cerevisiae* original (blue) and mutant (red) Rpb1 residues are indicated. The black striped lines show residues involved in the coordination of Mg^{2+} (metal A) and in the physical interaction with TFIIS. Independent alignments in A–D show the homology among eukaryotes (first row) and between eukaryotes, archaebacteria, and eubacteria (second row). No relevant homology was found for bacteria in D. Blue line, eukaryote; green line, archaebacteria; orange line, eubacteria; *, identical residue; :, conserved substitution; ., semiconserved substitution; Sc, *S. cerevisiae*; Sp, *Schizosaccharomyces pombe*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Pf, *Pyrococcus furiosus*; Ta, *Thermus aquaticus*; Ec, *Escherichia coli*.

all 11 TS alleles isolated in this screen were in the zinc-binding domain. Temperature-sensitive alleles of *RPB1* that involve changes in amino acids C67, C70, or H80 were previously isolated by directed mutagenesis (Donaldson and Friesen 2000).

Isolation of 6AU-sensitive *rpb1* mutants: We used the same library of mutagenized pL-RPB1 transformed into GRY3019 to isolate mutants sensitive to 6AU. Transformants were screened for the ability to grow in the presence of doxycycline, but for lack of growth in the presence of doxycycline plus 6AU. Among ~30,000 independent transformants analyzed in this screen, we found four $6AU^S$ mutations, *rpb1-D261N*, *rpb1-R320C*, *rpb1-E1103G*, and *rpb1-E1230K*. Two additional $6AU^S$ alleles, *rpb1-D260N* and *rpb1-N488D*, were obtained from a related screen of ~30,000 independent transformants done in a strain lacking TFIIS, GRY3100. The $6AU^S$ phenotype was reproduced after integration of the mutant alleles in the chromosome (Figure 2). As noted above, the *rpb1-E1230K* allele has been previously isolated as *rpo21-24*, a mutant with a reduced interaction of RNAPII with TFIIS (Wu *et al.* 1996). The other alleles are novel and alter highly conserved regions of Rpb1 (Figure 3) and are located in the vicinity of the RNA–DNA hybrid (Figure 4). Amino acids D260, D261, and R320 map in the lid and rudder domains of Rpb1. The lid and rudder are located in the upstream limit of the RNA–DNA hybrid and have been proposed to have a role in separating the RNA from the template strand (Cramer *et al.* 2001; Gnatt *et al.* 2001; Kettenberger *et al.* 2004; Westover *et al.* 2004). The mutants described here are the first eukaryotic mutants in those regions and their isolation as $6AU^S$ indicates that they are important for transcription *in vivo*. Amino acid N488 is located in the proximity of the invariant motif NADFDGD that coordinates one of two Mg^{2+} ions (metal A) in the active center of the enzyme downstream of the RNA–DNA hybrid (Cramer *et al.* 2001; Gnatt *et al.* 2001). N488 is also located remarkably close to the basic residues N445 and R446 in the RNAPII structure (Figure 4C). Mutations affecting N445 have a strong effect in transcription initiation, specifically in start site selection (Berroteran *et al.* 1994; Archambault *et al.* 1998). Amino acid E1103 is located near the position where the nontemplate strand is separated from the template strand downstream of the active site in a region defined by sequence homology (Jokerst *et al.* 1989) that has been shown to control the lateral mobility of the elongation complexes in bacteria (Bar-Nahum *et al.* 2005).

Altered regulation of *IMD2* and *SSM1* in the 6AU-sensitive *rpb1* mutants: Yeast cells respond to changes in

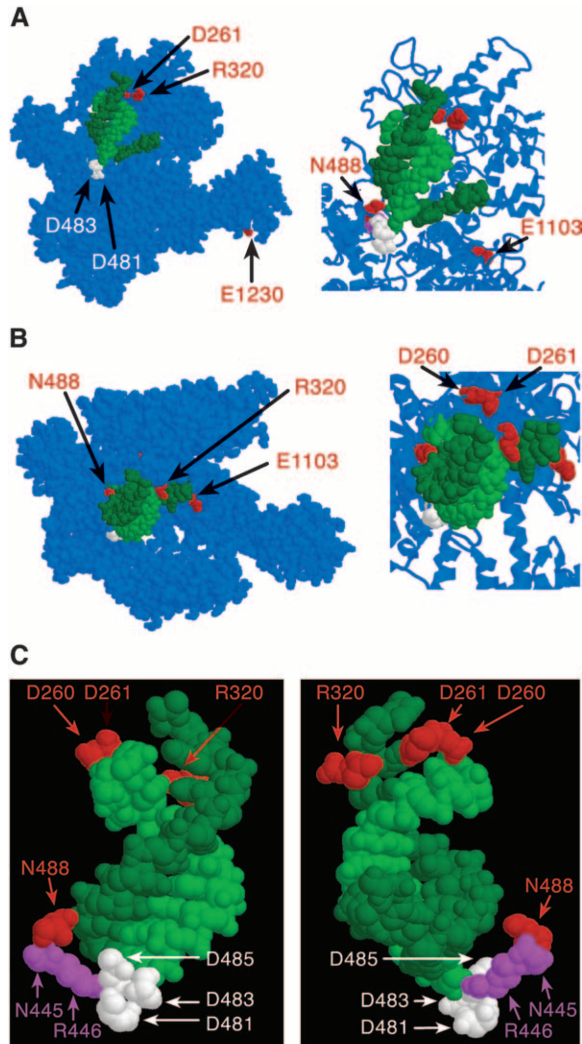


Figure 4.—The novel *rpb1* mutants sensitive to 6AU affect residues located in the vicinity of the RNA–DNA hybrid. Depiction of Rpb1 (blue) and the RNA–DNA hybrid (light–dark green) in a three-dimensional structure of the RNAPII transcription complex (1SFO.PDB) is shown. For simplicity other subunits of RNAPII are not shown. The position of the residues affected in the *rpb1* mutants sensitive to 6AU (red), the aspartic acids coordinating metal A (white), and two additional residues of interest (purple) are highlighted. (A) Right side view. (B) Top view. (C) Zoom showing the residues of interest closest to the RNA–DNA hybrid. Right and top are arbitrary but consistent with previous reports in the literature. The representation uses van der Waals spheres with the exception of the second pictures (zooms centered in the RNA–DNA hybrid) of A and B, where the Rpb1 backbone is represented by ribbons for better visualization of specific residues. The two pictures in C are rotated ∼180° in the *y*-axis. All the novel 6AU mutations alter residues in the internal surface of the RNAPII and are not in direct contact with any other subunit.

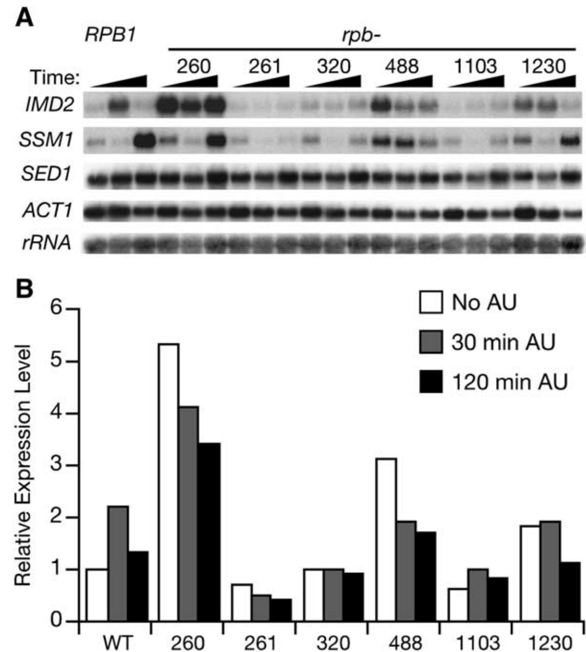


Figure 5.—*rpb1* alleles sensitive to 6-azauracil have altered transcription regulation of *IMD2* and *SSM1* genes. (A) Gene expression of *IMD2* and *SSM1* in response to 6AU determined by Northern analysis. *ACT1*, *SED1*, and *rRNA* are loading controls. For simplicity, *rpb1* mutant alleles are identified just by the position of the amino acid change. The triangles represent from left to right 0, 30, and 120 min in the presence of 6AU. (B) Averaged quantitation of two independent sets of RNA preparations and Northerns compared to the expression ratio of *IMD2* normalized to the expression of *SED1* in the absence of 6AU.

the pools of nucleosides caused by 6AU by inducing genes involved in nucleoside metabolism like *IMD2/PUR5* and *SSM1/SDT1*. Imd2 participates in the first step of the GMP anabolic pathway (Escobar-Henriques and Daignan-Fornier 2001) and Ssm1 is a pyrimidine nucleotidase required for detoxification of 6AU (Nakanishi and Sekimizu 2002). One reason cells can be sensitive to 6AU is because of a failure to induce these genes. Mutants that are sensitive to 6AU often fail to induce the *IMD2/PUR5* and *SSM1/SDT1* genes (Shaw and Reines 2000; Shimoaraiso *et al.* 2000). Therefore we tested the expression levels of *IMD2* and *SSM1* in our mutants and their response to 6AU. As loading controls, we also monitored the constitutively expressed *ACT1* and *SED1* genes and the level of RNA polymerase I-dependent rRNA25S ribosomal RNA. As shown in Figure 5, *IMD2* is clearly upregulated in *rpb1-D260N* and *rpb1-N488D* (and to a lesser extent in *rpb1-E1230K*) before the addition of 6AU. In other words, these mutants behave as if they are starved for nucleotides even in the absence of 6AU. In contrast, *rpb1-D261N*, *rpb1-R320C*, and *rpb1-E1103G* fail to induce *IMD2* or *SSM1* upon addition of 6AU. These results indicate an abnormal regulation of genes involved in nucleotide metabolism in the *rpb1* mutants tested and suggest that *rpb1* mutants can be sensitive to 6AU for very different reasons, perhaps reflective of defects in different steps of transcription.

***rpb1-N488D* decreases and *rpb1-E1103G* increases RNAPII polymerization rate *in vitro*:** To test the effect of the different amino acid substitutions on the biochemical

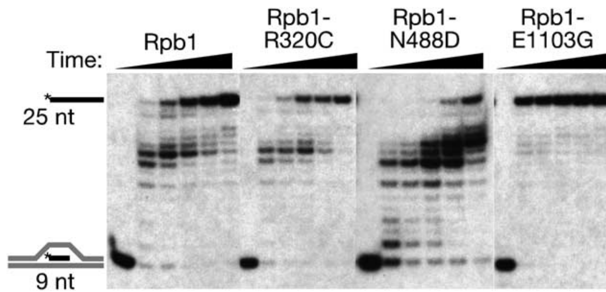
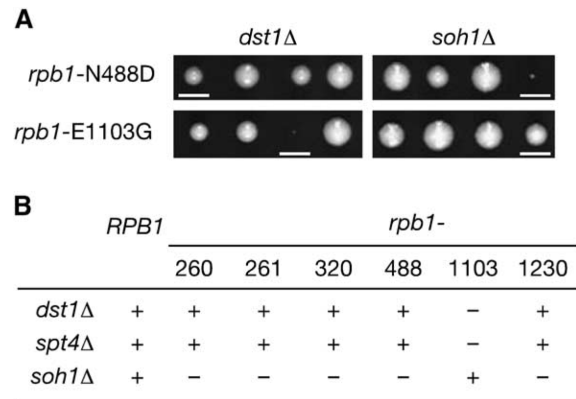


FIGURE 6.—RNAPII *in vitro* transcription rate is decreased and increased by the Rpb1 amino acid changes N488D and E1103G, respectively. RNAPII transcription complexes assembled *in vitro* elongate a short priming RNA upon addition of nucleotides. The Rpb1 amino acid change of the complexes is indicated. The solid line with an asterisk represents the RNA radioactively labeled at the 5′ end; the shaded lanes represent DNA strands; the triangles represent from left to right 0, 10, 20, 40, 90, and 180 sec in the presence of nucleotides.



	RPB1	rpb1-					
		260	261	320	488	1103	1230
dst1Δ	+	+	+	+	+	−	+
spt4Δ	+	+	+	+	+	−	+
soh1Δ	+	−	−	−	−	+	−

FIGURE 7.—*rpb1* alleles show differential synthetic phenotypes with transcription factor mutants. (A) Examples of tetratypes revealing the four meiotic products from selected crosses. The open bars indicate the double mutants *rpb1-N488D dst1* (top left tetrad), *rpb1-N488D soh1* (top right tetrad), *rpb1-E1103G dst1* (bottom left tetrad), and *rpb1-E1103G soh1* (bottom right tetrad). (B) Table showing the complete panel of growth phenotypes of the *rpb1* alleles in the absence of TFIIS (*dst1*), Spt4 (*spt4*), and Soh1 (*soh1*). For simplicity, *rpb1* mutant alleles are identified just by the position of the amino acid change.

properties of RNAPII, the mutations were integrated into strain GRY3030, which contains a hexahistidine-tagged Rpb3 that can be used for affinity purification of the RNAPII (KIREEVA *et al.* 2003). The mutant RNA polymerases were purified and the polymerization capabilities were tested *in vitro*, using factor-independent assembly of transcription elongation complexes (KIREEVA *et al.* 2003). TFIIS is not present or required in this assay and, as expected, *rpb1-E1230K* does not affect the polymerization properties of the RNAPII tested in this assay (not shown). We also did not detect a difference between RNAPII from wild type *vs.* that from *rpb1-D261N* (not shown) or *rpb1-R320C* in this assay (Figure 6). On the other hand, this assay clearly shows that the *rpb1-N488D* mutation causes a change in the intrinsic elongation properties of the enzyme, resulting in an RNAPII that is slower than the wild-type polymerase. In contrast, the RNAPII from *rpb1-E1103G* is faster than the wild-type enzyme. For unknown reasons, we were unable to introduce *rpb1-D260N* into GRY3030. These results again indicate that 6AU sensitivity can be caused by very different kinds of defects in RNAPII.

Synthetic interactions with *SOH1*, *SPT4*, and *DST1* distinguish 6AU-sensitive *rpb1* alleles *in vivo*: The $6AU^S$ mutants exhibited different biochemical defects and showed different alterations to the expression of *IMD2* and *SSM1*. To see whether these defects correlated with sensitivity to loss of specific transcription factors, we tested the genetic interaction of the $6AU^S$ *rpb1* alleles with the transcription elongation genes *DST1* and *SPT4* and with *MED31/SOH1*, a gene encoding a subunit of the Mediator transcription initiation complex (GUGLIELMI *et al.* 2004; LINDER and GUSTAFSSON 2004). The results, summarized in Figure 7, again show that these $6AU^S$ alleles have different defects. The *rpb1-E1230K* mutant, presumably due to its inability to interact with TFIIS, has a synthetic lethal phenotype with *soh1*, as expected on the basis of the synthetic lethal phenotype of *soh1 dst1* mutants (MALAGON *et al.* 2004). The novel mutants *rpb1-D260N*, *rpb1-D261N*, *rpb1-R320C*, and *rpb1-N488D* are also synthetic lethal with a *soh1* deletion, suggesting a role in transcription initiation or early elongation phases. In contrast, the *rpb1-E1103G* allele shows a synthetic lethal phenotype with *dst1* and *spt4*. Two other *rpb1* mutations, *rpb1-221* (H1367D) and *rpb1-244* (E1351K), isolated as suppressors of a cryosensitive *spt5* mutant, have been described as $6AU^S$ and synthetic with *dst1* (HARTZOG *et al.* 1998). The synthetic interaction of *rpb1-E1103G* with *DST1* and *SPT4* suggests an *in vivo* requirement of residue E1103 for a proper transcription elongation.

DISCUSSION

In this work we describe the isolation and initial characterization of novel *rpb1* mutations that are highly sensitive to the nucleotide-depleting drug 6AU. These mutants define several classes of defects that are distinguished by their behavior in an initiation factor independent *in vitro* transcription elongation assay, their regulation of genes involved in nucleotide metabolism induction (*IMD2* and *SSM1*), and their genetic interaction with mutants defective in elongation (*dst1* and *spt4*) or initiation (*soh1*). Three of the mutations (*rpb1-D260N*, *rpb1-D261N*, and *rpb1-R320C*) alter the lid or rudder loops that are postulated to have roles in separating the RNA–DNA hybrid and have a synthetic phenotype with *soh1*. Two of the mutants near the catalytic center (*rpb1-N488D*) and the homology box G downstream of the active site (*rpb1-E1103G*) alter the

intrinsic properties of RNAPII *in vitro* and show differential synthetic interactions *in vivo*.

Lid and rudder mutants: The lid and rudder loops of Rpb1, along with the Rpb2 fork loop 2, are located in the upstream limit of the RNA–DNA hybrid, forming a "strand-loop network" with dynamic complex interactions (Westover *et al.* 2004). Structural studies in yeast and bacteria indicate that these loops may facilitate the separation of the RNA from the template DNA strand (Korzheva *et al.* 2000; Westover *et al.* 2004). It has been suggested that the formation of the strand-loop network occurs during a transcriptional pause provoked by the clash of the 5′ end of the RNA with TFIIB during transcription initiation (Bushnell *et al.* 2004; Westover *et al.* 2004). This led to the proposal that during promoter escape the formation of the strand-loop network allows further chain elongation, causing a displacement of TFIIB in eukaryotes and of σ-factor in bacteria (Vassylyev *et al.* 2002; Westover *et al.* 2004). We show here *in vivo* phenotypes caused by alterations in the lid and rudder of RNAPII caused by the *rpb1-D260N*, *rpb1-D261N*, or *rpb1-R320C* mutations. Amino acids D260, D261, and R320 of Rpb1 in yeast and the correspondent amino acids in bacteria are located close to one another in the RNA polymerase crystal structures (Vassylyev *et al.* 2002; Westover *et al.* 2004) (see Figure 4C), suggesting possible roles in the formation of the strand-loop network. The *rpb1-D261N* and *rpb1-R320C* mutations cause a defect in the induction of *IMD2* and *SSM1* genes that may be sufficient to explain the sensitivity to 6AU. In contrast, the *rpb1-D260N* mutant expresses *IMD2* and *SSM1* even in the absence of 6AU. It will be interesting to determine whether these alterations in the expression levels of these genes involved in nucleotide metabolism reflect a direct effect on initiation or some indirect effect. We detected no defect in elongation efficiency for *rpb1-D261N* or *rpb1-R320C* in the transcription factor independent assay used here, which is consistent with the view that their defect is at some other step. Indeed, each of these lid and rudder mutants is unable to survive when combined with a defect in initiation caused by loss of a subunit of the Mediator initiation complex, *soh1*. Further experiments will be required to determine whether they are specifically defective in initiation and whether that defect is manifested at particular genes.

The active site mutant, *rpb1-N488D*: Treatment with 6AU alters the nucleotide pools and causes a decrease in the rate and processivity of RNAPII *in vivo* (Exinger and Lacroute 1992; Mason and Struhl 2005). Two mutations in RNAPII that decrease the RNA elongation rate *in vitro* have been described: the 6AU-sensitive *rpb2-10* allele in *S. cerevisiae* (Scafe *et al.* 1990; Powell and Reines 1996) and the *Drosophila melanogaster* C4 mutation corresponding to a change in *RPB1-R726* in yeast (Coulter and Greenleaf 1985). Similar to the 6AU treatment, Rpb2-P1018S (the *rpb2-10* mutation) decreases the polymerization rate and the processivity of RNAPII *in vivo* (Mason and Struhl 2005). A lower speed of RNAPII theoretically can increase the probability of transcriptional arrest, an irreversible state of RNAPII *in vitro* that can be rescued only by TFIIS and that has been invoked to explain transcription-associated recombination and mutation (Aguilera 2002). Surprisingly, although *rpb2-10* mutants show some synthetic interaction with *dst1*, as shown by the reduced levels of poly(A) RNA in a *rpb2-10 dst1* double mutant compared to the single mutations, *rpb2-10* mutants are not synthetic lethal with TFIIS (Lennon *et al.* 1998). Similarly, we found that Rpb1-N488D had a decreased RNA elongation rate *in vitro* and *rpb1-N488D* mutants were hypersensitive to 6AU and were not synthetic lethal with *dst1* on YEPD. We did note that the *rpb1-N488D dst1* double mutant showed slower growth than either single mutant on minimal media (data not shown). Similarly to the lid and rudder mutants, *rpb1-N488D* has a strong synthetic phenotype with *soh1*. Soh1 is a *bona fide* subunit of the transcription initiation Mediator complex (Guglielmi *et al.* 2004; Linder and Gustafsson 2004) originally isolated in a screen for suppressor of hyper-recombination mutants (Fan and Klein 1994). We believe that the simplest explanation for the synthetic interaction of *rpb1-N488D* with *soh1* is that, in addition to its possible role in transcription elongation highlighted by its similarities with *rpb2-10*, the Rpb1 residue N488 also plays a role in transcription initiation. This interpretation is supported by the fact that the *rpb1* mutations *sua8-1* (*rpb1-N445S*) and *sit1-278* (*rpb1-N445T*) alter amino acids that are located adjacent to N488 in the RNAPII structure (see Figure 4C) and affect transcription start site selection *in vivo* (Berroteran *et al.* 1994; Archambault *et al.* 1998). It remains to be determined whether the increased level of expression of *IMD2* and *SSM1* in the absence of 6AU caused by the *rpb1-N488D* mutation reflects an alteration in initiation.

The downstream mutant *rpb1-E1103G*: The $6AU^S$ mutant *rpb1-E1103G* causes an alteration in the regulation of *IMD2* and *SSM1* so that they fail to induce in response to 6AU. Rpb1-E1103G exhibited an increased RNA polymerization rate *in vitro* in our transcription factor independent assay. The position of the residue E1103 in the G loop, a region that has been suggested to modulate the catalytic activity of bacterial RNA polymerase, gives some insights into the effect of the mutation. Recently, Bar-Nahum and collaborators described a mutation in this G loop of bacterial RNA polymerase also showing an associated increase in the polymerization rate (Bar-Nahum *et al.* 2005). A "fast" RNAPII has also been described in *D. melanogaster*, the S1 mutant that altered the DNA–RNA hybrid-binding region of the Rpb2 homolog (Chen *et al.* 1996). Changes in the speed of the RNAPII may interfere with a series of tightly coupled mRNA processes occurring during elongation, as illustrated by the correlation

between RNAPII elongation rate and efficiency of mRNA splicing in eukaryotes (de la Mata *et al.* 2003; Howe *et al.* 2003). The *rpb1-E1103G* was unique in our collection in demonstrating a synthetic lethal interaction with the deletion of *DST1* or *SPT4*. Both TFIIS and the Spt4/Spt5 complex affect RNA splicing (Howe *et al.* 2003; Lindstrom *et al.* 2003; Xiao *et al.* 2005) and *rpb1* mutants synthetic with *dst1* were previously isolated by their genetic interaction with *SPT5* (Hartzog *et al.* 1998). Defects in mRNA processing caused by an increase in chain elongation rate may explain the high dependence on TFIIS and Spt4 for cell viability in the *rpb1-E1103G* mutant.

Conclusion: The collection of *rpb1* mutants described here, although originally isolated as sensitive to 6AU, exhibits several very different biochemical and genetic interaction phenotypes. RNAPII has multiple roles in transcription including initiation, promoter escape, elongation, splicing, transcription-coupled repair, and termination. The results presented here are consistent with the view that several of those roles can be rendered sensitive to nucleotide pool levels by mutations in different domains of Rpb1.

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