

Functional Interaction between Ssu72 and the Rpb2 Subunit of RNA Polymerase II in *Saccharomyces cerevisiae*

DONALD L. PAPPAS, JR., AND MICHAEL HAMPSEY*

Department of Biochemistry, Division of Nucleic Acids Enzymology, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

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SSU72 is an essential gene encoding a phylogenetically conserved protein of unknown function that interacts with the general transcription factor TFIIB. A recessive *ssu72-1* allele was identified as a synthetic enhancer of a TFIIB (*sua7-1*) defect, resulting in a heat-sensitive (Ts^-) phenotype and a dramatic downstream shift in transcription start site selection. Here we describe a new allele, *ssu72-2*, that confers a Ts^- phenotype in a *SUA7* wild-type background. In an effort to further define Ssu72, we isolated suppressors of the *ssu72-2* mutation. One suppressor is allelic to *RPB2*, the gene encoding the second-largest subunit of RNA polymerase II (RNAP II). Sequence analysis of the *rpb2-100* suppressor defined a cysteine replacement of the phylogenetically invariant arginine residue at position 512 (R512C), located within homology block D of Rpb2. The *ssu72-2* and *rpb2-100* mutations adversely affected noninduced gene expression, with no apparent effects on activated transcription in vivo. Although isolated as a suppressor of the *ssu72-2* Ts^- defect, *rpb2-100* enhanced the transcriptional defects associated with *ssu72-2*. The Ssu72 protein interacts directly with purified RNAP II in a coimmunoprecipitation assay, suggesting that the genetic interactions between *ssu72-2* and *rpb2-100* are a consequence of physical interactions. These results define Ssu72 as a highly conserved factor that physically and functionally interacts with the RNAP II core machinery during transcription initiation.

Eukaryotic RNA polymerase II (RNAP II) is a multisubunit enzyme that is responsible for transcription of all protein-encoding genes. *Saccharomyces cerevisiae* RNAP II is a 12-subunit complex encoded by the genes *RPB1* to *RPB12* (reviewed in references 2, 53, and 55). The two largest subunits, Rpb1 and Rpb2, are homologous to the β' and β subunits of bacterial RNA polymerase (RNAP), respectively (23, 49). The yeast counterpart of the bacterial α subunit appears to be shared between the Rpb3 and Rpb11 subunits (26, 57). Accordingly, Rpb1, Rpb2, Rpb3, and Rpb11 comprise the functional equivalent of the bacterial $\alpha_2\beta\beta'$ core RNAP. The functions of the remaining RNAP II subunits are less clear, although five subunits, Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12, are shared among all three forms of RNAP and therefore function in transcription of all genes. With the exception of Rpb4 and Rpb9, all subunits are essential for cell viability. Rpb4 forms a subcomplex with Rpb7 that is required for promoter-specific initiation but is dispensable for elongation (13).

RNAP II is unable to initiate promoter-specific transcription on its own. Promoter recognition requires the general transcription factors (GTFs), which include the TATA binding protein (TBP), TFIIB, TFIIE, TFIIIF, and TFIIF (reviewed in references 17 and 33). TBP nucleates assembly of a transcription preinitiation complex by binding the TATA element and inducing a sharp bend in the DNA template. TFIIB binds the TATA-TBP complex and is responsible for defining the polarity of transcription by binding asymmetrically to the BRE element, located immediately upstream of the TATA box (28, 50). RNAP II, in association with TFIIIF, binds the TATA-TBP-TFIIB ternary complex, followed by TFIIE and TFIIF. TFIIF is a multisubunit complex with catalytic activities responsible for phosphorylation of the carboxy-terminal repeat domain of

the Rpb1 subunit of RNAP II, promoter melting and promoter clearance (25).

The GTFs were discovered based on their requirement for accurate initiation in vitro (29, 52) and are highly conserved among eukaryotic organisms (reviewed in references 17, 30, 33, and 38). Furthermore, the TBP and TFIIB requirements for transcription predate the divergence of Eukarya and Archaea (reviewed in reference 5). Nonetheless, the RNAP II requirement for GTFs is not universal but is instead dictated by promoter sequence and architecture (16, 35, 51). Indeed, genome-wide expression analysis revealed that most components of the RNAP II transcriptional machinery are dispensable for expression of subsets of genes (21). Conversely, since only a limited number of promoters have been analyzed in vitro, it is conceivable that additional GTFs might be identified based on their requirement for accurate initiation from specific promoters. Other factors might be identified based on their ability to stimulate activator-independent transcription. For example, the yeast Tsp1 (Sub1) protein was identified as a positive effector of in vitro transcription in the absence of a sequence-specific activator (20). Tsp1 interacts physically and genetically with TFIIB and is homologous to the human transcriptional cofactor PC4 (20, 27). Additional basal factors are likely to be identified, either by their requirement for promoter-specific initiation, by their ability to stimulate activator-independent transcription, or by genetic interactions with GTFs or RNAP II subunits.

The yeast gene encoding TFIIB (*SUA7*) was identified as an effector of transcriptional accuracy (36). Replacements at either of two phylogenetically invariant residues, glutamate-62 (E62) or arginine-78 (R78), caused a marked start site shift downstream of the normal site at the *CYC1* and *ADH1* promoters (37). Nearly identical effects on transcriptional accuracy are conferred by the *sua8* alleles of *RPB1*, suggesting a functional interaction between TFIIB and Rpb1 during start site selection (6). In an effort to identify other factors that affect initiation, we isolated suppressors of the *sua7-1* (E62K)

* Corresponding author. Mailing address: Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Phone: (732) 235-5888. Fax: (732) 235-5889. E-mail: hampsemi@umdnj.edu.

mutation. The *ssu71* and *ssu73* suppressors encode altered forms of the largest subunit of TFIIF (Tfg1) and the Rpb9 subunit of RNAPII, respectively (46, 47). In contrast, *ssu72* was identified as an enhancer of the TFIIB E62K defect: the *ssu72-1* allele confers a heat-sensitive (Ts^-) growth defect and a dramatic downstream start site shift, with both effects being dependent upon the *sua7-1* allele (48).

The *SSU72* gene is essential for cell viability and encodes a novel protein of undefined function (48). To further characterize *Ssu72*, we generated a new *ssu72* allele that confers a tight Ts^- phenotype, independent of a TFIIB defect. We have taken advantage of the *ssu72* Ts^- phenotype to isolate a new *rpb2* mutation encoding an altered form of the Rpb2 subunit of RNAP II. The results presented here demonstrate that *Ssu72* is a transcription factor that interacts with the core RNAP II machinery both in vivo and in vitro.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study were derived from LRB555 (*MATA his3Δ200 leu2-3,112 ura3-52*). YZS84 is isogenic to LRB555, except for the *ssu72-2* allele, which was generated by site-directed mutagenesis and subsequent allele replacement, as described below. YDP19 (*ssu72-2 rpb2-100 shs2-1*) is a spontaneous Ts^+ revertant of YZS84; YDP135 (*ssu72-2 rpb2-100*) is a segregant derived from a cross of YDP19 with YDP87 (*ssu72-2 RPB2 SHS2*). The plasmid-shuffle strain YZS89A (*ssu72::LEU2 [SSU72-URA3]*) was described previously (48).

Growth media, genetic methods, and phenotypes. All growth media were prepared according to standard recipes (43). $-Ino$ medium is synthetic complete medium lacking inositol; $+Ino$ control medium contains 55 μ M inositol. Standard yeast genetic methods were used for making crosses, selecting diploids, inducing sporulation, and dissecting tetrads (44). The following designations are used for phenotypes: Ts^- , temperature sensitivity, defined by impaired growth on YPD medium at 37°C; Cs^- , cold sensitivity, defined by impaired growth on YPD medium at 16°C; and Ino^- , impaired growth on $-Ino$ medium at 30°C relative to growth on $+Ino$ control medium. Plasmids carrying the *URA3* marker were counterselected on synthetic medium containing 5-fluoro-orotic acid (8).

Construction of the *ssu72-2* mutant. The *ssu72-2* allele was constructed by site-directed mutagenesis as described previously (48) using synthetic oligonucleotide oZS-200 (5'-GAAGATTTGATGAATGCAAGGTGGGAAATTAAC-3'). The underlined GC bases of the GCA triplet encode alanine at amino acid position 129. The *EcoRI-KpnI* DNA fragment encompassing the *ssu72-2* open reading frame (ORF) was cloned into the corresponding sites of plasmid pRS314 (45), creating the *ssu72-2 TRP1 CEN* plasmid pM721. The phenotype of *ssu72-2* was initially determined by plasmid shuffle using host strain YZS89A.

Allele replacement of the normal *SSU72* chromosomal locus was made by integration-excision (40). The *EcoRI-KpnI* fragment encompassing *ssu72-2* was cloned into the corresponding sites of plasmid pRS306 (45), yielding the *ssu72-2*-integrating plasmid pM728. pM728 was linearized at the unique *Clal* site located with the *SSU72* coding region and introduced into strain LRB555. Ura^+ transformants were isolated and subsequently counterselected on 5-fluoro-orotic acid medium. Strains retaining the *ssu72-2* allele were identified based on the Ts^- phenotype associated with this allele (see above) and confirmed by the ability of the Ts^- phenotype to be complemented by plasmid-borne *SSU72*. Southern blot analysis confirmed integration and excision at the *SSU72* locus (D. L. Pappas, Jr., unpublished results).

Isolation and sequence analysis of the *rpb2-100* allele of *RPB2*. The *rpb2-100* allele of *RPB2* was recovered by gap repair (40). Plasmid RY2119 (*RPB2 URA3 CEN*) was digested to completion with *Sna*BI, which removes most of the *RPB2* ORF. Vector DNA flanked by *RPB2* sequences was isolated by agarose gel electrophoresis, purified, and introduced into strain YDP135 (*rpb2-100*). Ura^+ colonies were selected and subsequently screened for retention of the Cs^- and Ino^- phenotypes conferred by the *rpb2-100* allele of *RPB2*. Plasmid DNA was recovered, amplified in *Escherichia coli*, and analyzed by restriction digestion to confirm gap repair by the *rpb2-100* locus. This resulting plasmid, pDP73, failed to rescue the Cs^- and Ino^- phenotypes when introduced into strains YDP19 and YDP135, confirming recovery of *rpb2-100*. To facilitate sequence analysis, the 1.88-kb *KpnI-EcoRI*, 1.05-kb *EcoRI-Bgl*II, and 2.5-kb *PstI-SalI* *rpb2-100* fragments from pDP73 were cloned into the *KpnI-EcoRI* sites of pRS316, *EcoRI-Bam*HI sites of pRS316, and *PstI-SalI* sites of pRS424, respectively. Single-stranded DNA was generated using VCS-M13 helper phage in the presence of kanamycin as described previously (41). The DNA sequence of the entire *RPB2* ORF was determined using an ABI Prism automated DNA sequencer. DNA sequence comparisons were performed using the BLAST algorithm accessed via the Saccharomyces Genome Database (<http://genome-www.stanford.edu/Saccharomyces/>).

RNA analyses. *CYC1* transcript levels were analyzed by S1 nuclease protection. Strains were grown at 30°C in synthetic complete medium containing 2%

glucose to mid-log phase (optical density at 600 nm [OD_{600}] = 0.6). Cells were collected, washed, and used to inoculate two 50-ml cultures of synthetic complete medium containing either 2% glucose or 3% glycerol plus 2% ethanol as the sole carbon sources. Cultures were incubated at 30°C for 8 h, and cells were harvested. Total RNA was isolated and S1 nuclease protection assays were performed as described previously (22), using synthetic oligonucleotides 5'-GTGT AGCACCTTTCTTAGCAGAACC GGCCCTTGAATTCAGTCGACGG and 5'-GGAATTTCCAAGATTAAATTGGAGTCGAAAGCTCGCCTTA for *CYC1* and tRNA^W, respectively.

INO1 transcript levels were analyzed by Northern blotting. Cells were grown at 30°C in synthetic complete medium to mid-log phase, collected by centrifugation, washed, and used to inoculate two 50-ml cultures of synthetic complete medium either lacking or containing inositol (55 μ M). Cultures were incubated at 30°C for 8 h, and cells were harvested. Total RNA was extracted and analyzed as described previously (36). The *INO1* probe was generated by nick translation of a 1.0-kb *INO1 EcoRI-HindIII* fragment derived from plasmid pJM310 in the presence of [α -³²P]dCTP. The U6 control probe was a nick-translated 450 bp fragment encompassing the *SNR6* gene. *CYC1* and *INO1* transcript levels were quantified using the National Institutes of Health Image program (<http://rsb.info.nih.gov/nih-image/>) and normalized to the tRNA^W and U6 RNA loading control signals, respectively.

β -Galactosidase assays. Strains harboring *CYC1-lacZ* (pLG265-UP1 [15]), *INO1-lacZ* (pJS325 [42]), or *PGKI-lacZ* (pN1086 [7]) reporter plasmids were grown at 30°C in $-Ura$ medium to mid-log phase (OD_{600} = 0.6). Cells were harvested and resuspended in $-Ura$ medium containing either (i) 2% glucose or 3% glycerol plus 2% ethanol for *CYC1-lacZ* strains, (ii) synthetic complete medium either containing or lacking 55 μ M inositol for *INO1-lacZ* strains, or (iii) no addition for *PGKI-lacZ* strains. Cells were incubated for 8 h, harvested by centrifugation, and resuspended in 500 μ l of breaking buffer (100 mM Tris-HCl [pH 8], 1 mM dithiothreitol, 20% glycerol; 2 mM phenylmethylsulfonyl fluoride). Cell extracts were prepared by vortexing with 0.5-mm-diameter glass beads six times in 15-s bursts. β -Galactosidase assays were done as described previously (24). Activities were determined by duplicate assays of three independent transformants and are expressed according to the following formula: (1.7 ml \times OD_{420} units)/(0.0045 \times cell extract volume [milliliters] \times reaction time [minutes] \times protein concentration [milligrams per milliliter]).

In vitro transcription and translation reactions. Radiolabeled TBP, TFIIB, and *Ssu72* were generated using the TNT kit (Promega) in the presence of [³⁵S]methionine according to vendor specifications. Template DNAs were purified using a Mini-Prep kit (Qiagen). Plasmids pT7-IID (9) and pET-SUA7 were used as templates for synthesis of TBP and TFIIB, respectively. *SSU72* and *ssu72* allelic DNAs were amplified by PCR using oligonucleotides 5'-GGAATTTCCA TATGCTAGTCATCGC and 5'-CGCGGATCCTTAGTGATGGTGATGGT GATGTTTGTAATATGAAGGAGCG. This amplifies the *SSU72* ORF with sequences coding for a hexahistidine tag at the C terminus and flanking *NdeI* and *Bam*HI sites (underlined). The PCR products were digested and ligated into the *NdeI* and *Bam*HI sites in pCITE-4a(+) (Novagen), generating the *ssu72* templates pM1540 (*SSU72*), pM1541 (*ssu72-1*), pM1542 (*ssu72-2*), or pM1543 (*ssu72-4*).

RNAP II immunoprecipitation. Immunoprecipitation assays were performed as described previously (34), with minor modifications. Highly purified yeast RNAP II (425 ng) and in vitro-transcribed and translated input proteins (10 μ l) were incubated in 250 μ l of P100 buffer (50 mM Tris-acetate [pH 7.9], 10% glycerol, 100 mM potassium acetate, 14 mM magnesium acetate, 4 mM dithiothreitol, 1 mM EDTA, 50 μ g of bovine serum albumin per ml, and protease inhibitors [0.5 μ g of pepstatin A per ml, 0.5 μ g of leupeptin per ml, 2 μ g of chymostatin per ml, 2.5 μ g of antipain per ml, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamide]) at 4°C for 18 h. Pansorbin (Calbiochem) was harvested by centrifugation and resuspended in blocking buffer (50 mM Tris-acetate [pH 7.9], 100 mM potassium acetate, 25 mg of PVP-40 [Sigma] per ml and 30 mg of bovine serum albumin per ml [pH 7.9]). Following incubation for 20 min at room temperature, Pansorbin was centrifuged and resuspended to its original volume in P100 buffer. Twenty-five microliters of preblocked Pansorbin was added to each reaction mixture. Samples were incubated at 4°C for 30 min and centrifuged at 15,000 rpm for 2 min. Supernatants were transferred to fresh tubes containing 5 μ g of anti-RNAP II monoclonal antibody (8WG16; Covance, Inc.) and incubated for 90 min at 4°C. Preblocked Pansorbin (25 μ l) was added, and the reaction mixtures were incubated for 60 min at 4°C. The samples were centrifuged at 16,000 \times g for 2 min. The pellets were washed three times with 250 μ l of P100 buffer containing 0.1% NP-40 and once with 250 μ l of P100 buffer. Pellets were resuspended in 40 μ l of sample buffer, and 20 μ l of each was resolved in a sodium dodecyl sulfate-12% polyacrylamide gel. Following electrophoresis, the gel was treated with En³Hance (Dupont NEN), dried, and exposed to a phosphor screen. Products were visualized using a PhosphorImager and ImageQuant software (Molecular Dynamics).

RESULTS

The *Ssu72* protein is phylogenetically conserved. *Ssu72* was reported to be a novel protein with no apparent homologs in the databases (48). Genome sequencing projects have since

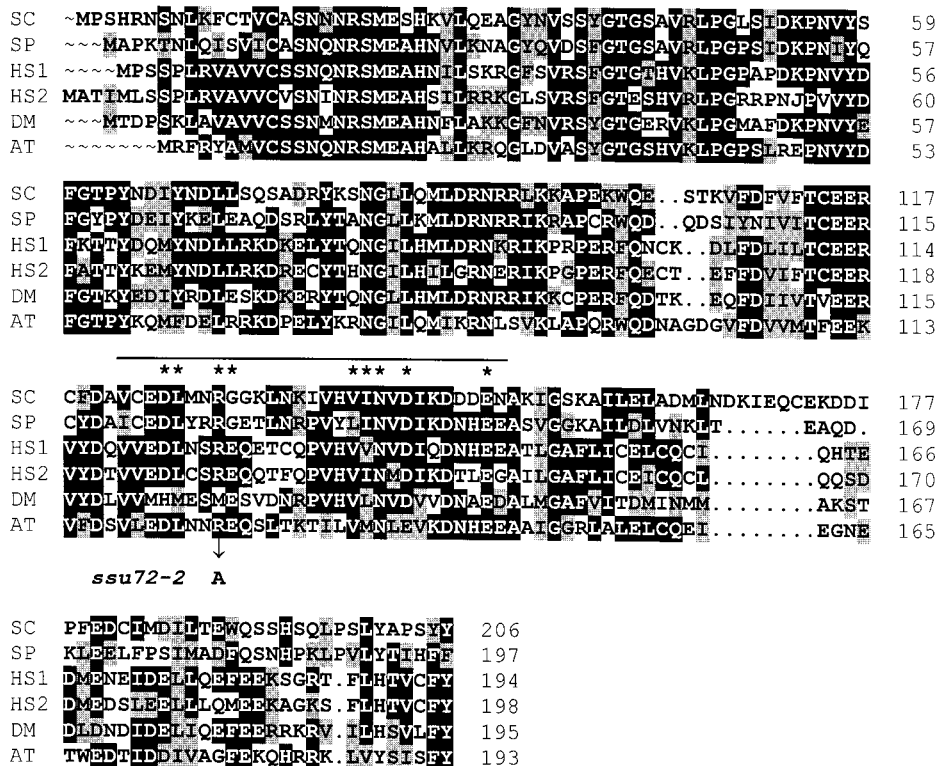


FIG. 1. Sequence similarities among eukaryotic Ssu72 proteins. The Ssu72 proteins from *S. cerevisiae* (SC), *S. pombe* (SP), human (HS1 and HS2), fruit fly (DM), and *A. thaliana* (AT) were identified using the BLAST algorithm (1) and aligned using the University of Wisconsin Genetics Computer Group programs PILEUP and BOXSHADE. Identical and similar amino acid residues are highlighted in black and gray, respectively. The overbar encompassing residues 122 to 150 of *S. cerevisiae* Ssu72 indicates the region of sequence similarity to ATP-dependent RNA helicases noted previously (48). The asterisks indicate residues within this region that are phylogenetically invariant among the helicases. The *ssu72-2* allele encodes an alanine replacement of the invariant arginine at position 129 (R129A).

identified homologs of Ssu72 in *Schizosaccharomyces pombe*, human, *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Caenorhabditis elegans* cells. Indeed, two distinct homologs of Ssu72 have been found in human cells. The yeast, human, fly, and plant proteins are all approximately 200 residues in length and exhibit 46 to 54% sequence identity (BLAST values of $<e^{-39}$) to *S. cerevisiae* Ssu72 (Fig. 1). The *C. elegans* protein is much larger, comprising 1,357 residues, although the region of sequence similarity is confined to the N terminus, showing 43% identity to *S. cerevisiae* Ssu72 (data not shown). No proteins with structural similarity to Ssu72 were found among the bacterial or archaeal protein sequence databases, suggesting that Ssu72 function is common to eukaryotes but is not conserved in either eubacteria or archaea.

Construction of the *ssu72-2* mutant. The original *ssu72* allele (*ssu72-1*) enhances the *sua7-1* defect by conferring a Ts^- growth defect and by dramatically shifting transcription initiation downstream of the normal site at the *ADHI* gene (48). These are pronounced effects but are dependent upon the *sua7-1* mutation. Indeed, the *ssu72-1* mutation confers no apparent phenotype in a *SUA7* wild-type background.

As part of our efforts to characterize *SSU72*, we sought an *ssu72* mutant that exhibits phenotypes independent of *sua7-1*. The Ssu72 amino acid sequence includes a region, encompassing residues 122 to 150, that is highly conserved among ATP-dependent RNA helicases (Fig. 1). Although other sequences common to this class of helicases, including the DEAD box and HRIGRXXXR motif, are not found in Ssu72, all of the invariant residues within the conserved region are present in

Ssu72 (Fig. 1). Since *SSU72* is essential for cell viability, we reasoned that replacement of a conserved amino acid within this region might confer a growth phenotype. Accordingly, we constructed an allele, *ssu72-2*, that encodes an alanine replacement of the invariant arginine at position 129 (R129A). Using a plasmid-shuffle assay, we found that the *ssu72-2* mutant was viable but failed to grow at 37°C. The plasmid-borne *ssu72-2* allele was then used to replace the chromosomal copy of *SSU72*, yielding strain YZS84. Consistent with the phenotypes associated with the plasmid-borne *ssu72-2* mutation, YZS84 was viable but distinctly Ts^- (Fig. 2).

Genetic suppression of *ssu72-2*. To identify factors that interact with Ssu72, we isolated suppressors of the *ssu72-2* Ts^- defect. Forty spontaneous Ts^+ revertants of strain YZS84 were isolated at 37°C. When scored for pleiotropic phenotypes, four revertants failed to grow in the absence of inositol yet grew normally on synthetic complete medium. This phenotype is denoted Ino^- and is often associated with defects in components of the general transcriptional machinery (18). Two of the Ino^- revertants were also cold sensitive (Cs^-), growing poorly on YPD medium at 16°C. One of these strains, YDP19, exhibited distinct $Ts^+ Cs^- Ino^-$ phenotypes (Fig. 2) and was chosen for further characterization.

Strain YDP19 ($Ts^+ Cs^- Ino^-$) was backcrossed with strain YDP87 ($Ts^- Cs^+ Ino^+$), and the resulting diploid strain was phenotypically $Ts^- Cs^+$ and Ino^+ , indicating that all YDP19 phenotypes are the result of a recessive mutation(s). When this strain was sporulated and dissected (11 four-spore tetrads), the $Ts^+ : Ts^-$ phenotypes segregated 0:4, 1:3, or 2:2, whereas the

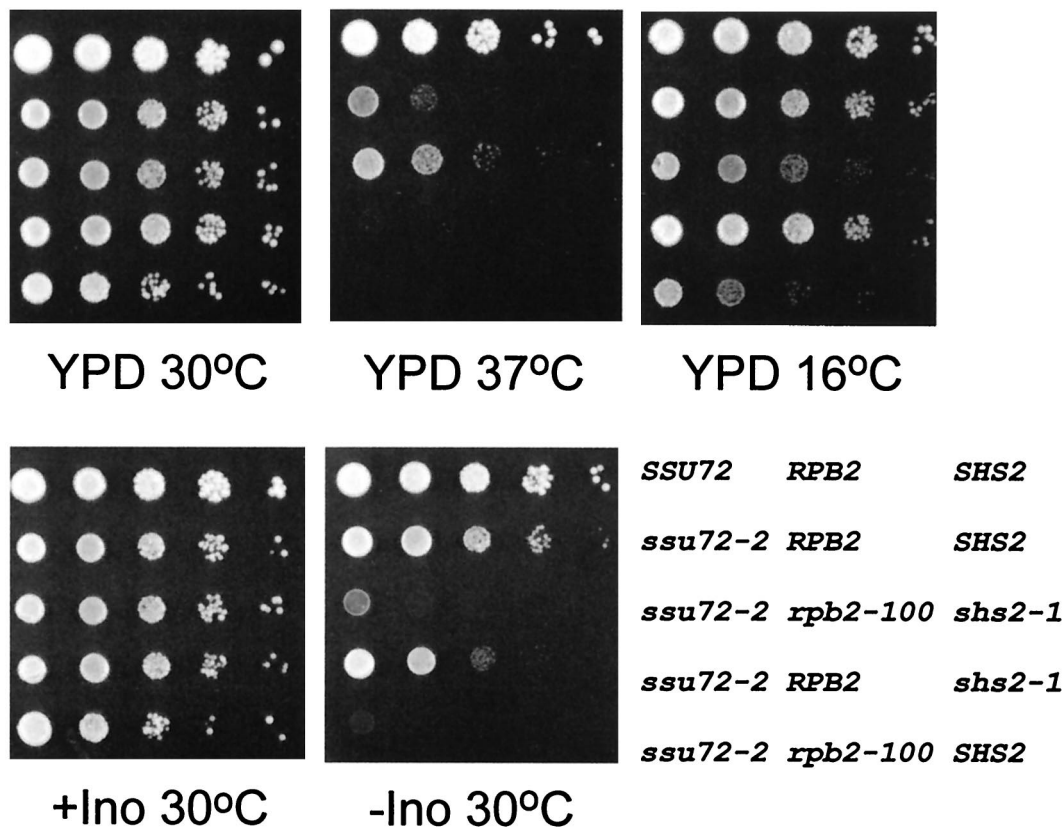


FIG. 2. Growth defects associated with *ssu72*, *rpb2-100*, and *shs2* mutations. Relevant genotypes are indicated for the wild-type strain LRB535 (*SSU72 SHS1 SHS2*), primary mutant YZS84 (*ssu72-2 RPB2 SHS2*), revertant YDP19 (*ssu72-2 rpb2-100 shs2-1*), a YDP19 derivative carrying a plasmid-borne copy of *RPB2* (*ssu72-2 RPB2 shs2-1*), and the YDP135 segregant (*ssu72-2 rpb2-100 SHS2*). The complete genotypes of these strains and the growth media are defined in Materials and Methods.

Cs⁺:Cs⁻ and Ino⁺:Ino⁻ phenotypes segregated 2:2. Furthermore, all Ts⁺ segregants were Cs⁻ and Ino⁻. These data indicate that suppression of the *ssu72-2* Ts⁻ phenotype is the result of mutations in two unlinked genes and that mutation in one of these genes confers the Cs⁻ and Ino⁻ phenotypes. We tentatively designated these two suppressor genes *shs1-1* (*rpb2-100* [see below]) and *shs2-1*. The *shs1-1* allele is responsible for the Cs⁻ and Ino⁻ phenotypes, whereas *shs2-1* confers no pronounced phenotype in the absence of *shs1-1*.

Identification of the *shs1-1* suppressor. We exploited the Cs⁻ and Ino⁻ phenotypes of *shs1-1* to clone the wild-type gene from a YCp50 genomic library (39). From approximately 90,000 Ura⁺ transformants of YDP135 (*ssu72-2 shs1-1*), 25 Cs⁺ strains were isolated, 4 of which were restored to Ino⁺. Plasmid DNAs were isolated from each of these four transformants and were found to include overlapping DNA inserts within the YCp50 vector. One plasmid, pDP99, was reintroduced into YDP135, and all of the scored transformants were Cs⁺ and Ino⁺, indicating that complementation of the Cs⁻ and Ino⁻ phenotypes was a consequence of plasmid DNA rather than strain reversion.

Restriction analysis of plasmid pDP99 defined an insert of approximately 8.0 kb in the YCp50 vector. Partial DNA sequence analysis identified a segment of the yeast genome from chromosome XV encompassing *RPB2*, the gene encoding the second-largest subunit of RNAP II. To further define the relationship between *shs1-1* and *RPB2*, plasmid RY2119, which includes the entire *RPB2* coding region but neither of the flanking ORFs of the original clone, was introduced into

YDP135. The resulting transformants were phenotypically Ts⁻, Cs⁺, and Ino⁺, confirming that *RPB2*, rather than a flanking gene, complements the suppressor mutation. To confirm that the suppressor is allelic to *RPB2*, the *URA3* gene was integrated by homologous recombination adjacent to the *RPB2* locus of strain YDP87. The resulting strain (*ssu72-2 ura3 RPB2-URA3*) was crossed with YDP19 (*ssu72-2 ura3 shs1-1 shs2-1*), and a diploid strain was sporulated and dissected. A total of 44 segregants were obtained and scored. All Ura⁺ progeny were phenotypically Cs⁺ and Ino⁺, and all Ura⁻ progeny were Cs⁻ and Ino⁻. Thus, the suppressor segregates opposite to *RPB2*, thereby confirming that *shs1-1* is allelic to *RPB2*. Accordingly, we have renamed the *shs1-1* suppressor *rpb2-100*.

The *rpb2-100* mutation. The *rpb2-100* allele was cloned by gap repair, and the entire ORF was sequenced using a collection of primers that span the *RPB2* ORF. A single-base-pair substitution at position 1534 (C1534T) was identified, encoding replacement of arginine-512 by cysteine (R512C). The Rpb2 subunit of RNAP II is structurally conserved among eukaryotic, archaeal, and bacterial RNAPs, including eukaryotic RNAPs I, II, and III. Sequence comparisons identified nine blocks, designated A to I, which are highly conserved among all RNAPs (49). R512 is the first amino acid within homology block D and is phylogenetically invariant (Fig. 3). The results presented here indicate that R512, despite its phylogenetic invariance, is not essential for cell viability. Indeed an *rpb2-100* allele in an otherwise normal (*SSU72*⁺) genetic back-

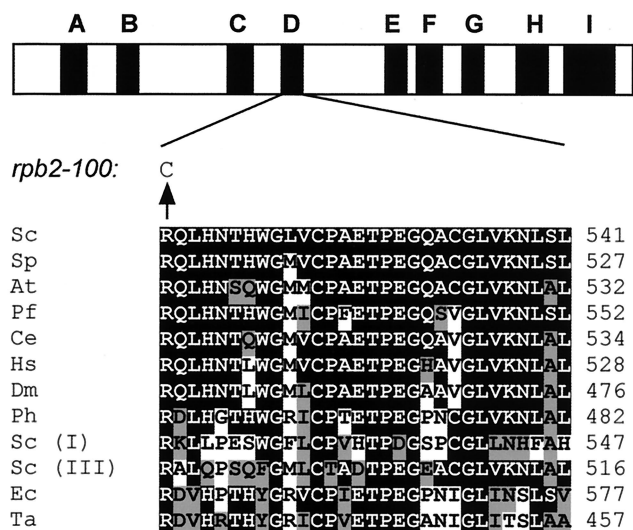


FIG. 3. The *rpb2-100* mutation. The *rpb2-100* allele of *RPB2* encodes an R512C replacement at a phylogenetically invariant position within homology block D of Rpb2. The alignment of homology block D (30 residues) is shown for *S. cerevisiae* (Sc), *S. pombe* (Sp), *A. thaliana* (At), *Plasmodium falciparum* (Pf), *C. elegans* (Ce), *Homo sapiens* (Hs), *Drosophila melanogaster* (Dm), *Pyrococcus horikoshii* (Ph), the second-largest subunits (Rpa135 and Rpc128) of RNAP I and RNAP III of *S. cerevisiae* [Sc (I) and Sc (III), respectively], and the β subunits of *E. coli* (Ec) and *T. aquaticus* (Ta) RNAPs. Amino acids are indicated by the single-residue code; the numbers at the right indicate the position of the C-terminal residue of block D.

ground displays only weak growth impairment at 30°C but is severely Cs^- and Ino^- (data not shown).

The *ssu72-2* and *rpb2-100* alleles affect noninduced transcription in vivo. The effects of *ssu72-2* and *rpb2-100* mutations on transcription from specific promoters were determined in vivo using the wild-type strain (LRB535), an *ssu72-2* primary mutant (YZS84), an *ssu72-2 rpb2-100 shs2-1* suppressor strain (YDP19), and an *ssu72-2 rpb2-100* segregant (YDP135). Effects on *CYC1* expression are presented in Fig. 4A. Under repressing conditions (2% glucose), the *CYC1* transcript level was reduced in the *ssu72-2* mutant to 42% of normal (compare lanes 1 and 3). Expression was further diminished in the *ssu72-2 rpb2-100 shs2-1* revertant to 17% of normal (lane 5), an effect that could be attributed to *rpb2-100* rather than *shs2-1*, since the same level of expression was observed in the *ssu72-2 rpb2-100* mutant (lane 7). These effects occur at the level of noninduced transcription, since all strains retained the ability to respond to derepressing conditions.

The effects on *CYC1* transcription were confirmed using a *CYC1-lacZ* reporter. In the wild-type strain 1,300 U of activity was measured under repressing conditions, and this activity was enhanced 3.5-fold under derepressing conditions (Fig. 4B). Noninduced expression was diminished 9-fold in the *ssu72-2* primary mutant (140 U) and was reduced an additional 30-fold in the *ssu72-2 rpb2-100 shs2-1* suppressor strain (4.8 U). The *shs2-1* allele did not contribute to this effect, since activity was equally low in an *ssu72-2 rpb2-100* strain (3.3 U). Each mutant responded to derepressing conditions, although the absolute activities were diminished in parallel with effects on basal activity. These results are consistent with the RNA analyses (Fig. 4A), confirming that *ssu72-2* and *rpb2-100* diminish noninduced transcription from the *CYC1* promoter.

INO1 expression was determined by Northern blot analysis (Fig. 5A). Strong induction of *INO1* mRNA was observed in

the wild-type strain (compare lanes 1 and 2). Induced levels were diminished to approximately 70% of normal in the *ssu72-2* mutant (lane 4) and were further reduced to 43% of normal in the *ssu72-2 rpb2-100 shs2-1* suppressor strain (lane 6). Interestingly, *rpb2-100* had a more dramatic effect in the absence of *shs2-1*, nearly eliminating *INO1* expression (lane 8). These results are consistent with the effects of *ssu72-2* and *rpb2-100* on *INO1-lacZ* reporter gene expression under repressing conditions (Fig. 5B). Although the Northern analysis does not distinguish between effects on noninduced versus

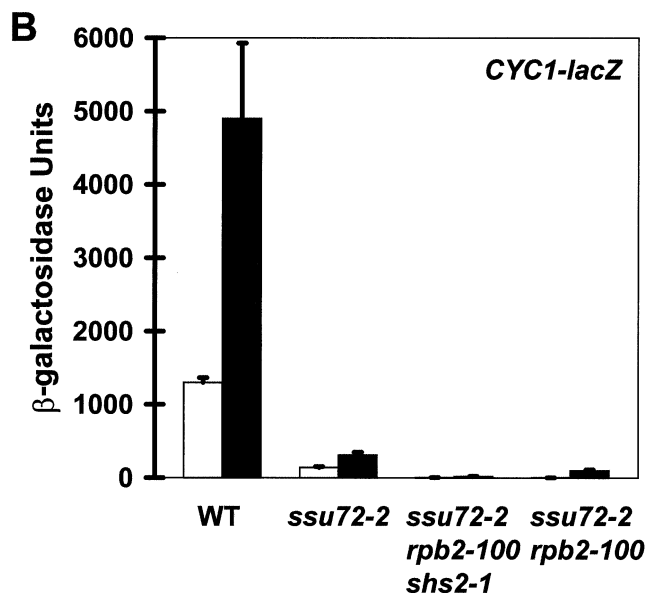
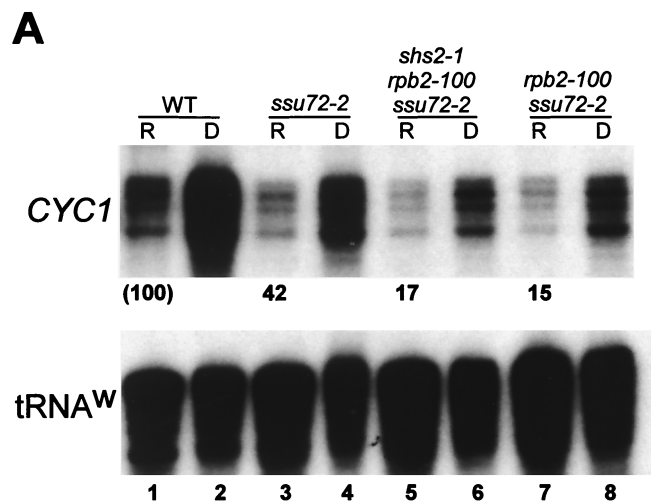


FIG. 4. Effects of *ssu72* and *shs* mutations on *CYC1* expression. (A) The top panel indicates *CYC1* transcript levels, determined by S1 nuclease protection, for strains grown under repressing (2% glucose) (lanes R) and derepressing (3% glycerol and 2% ethanol) (lanes D) conditions. Strains are LRB535 (wild type [WT]), YZS84 (*ssu72-2*), YDP19 (*ssu72-2 rpb2-100 shs2-1*), and YDP135 (*ssu72-2 rpb2-100*). Transcript levels under repressing conditions are presented as percentages, normalized to the level for the wild-type strain. The bottom panel is a loading control and indicates *tRNA^W* levels. (B) β -Galactosidase activities expressed from the *CYC1-lacZ* plasmid pLG265-UP1. Activity units under repressing and derepressing conditions, respectively, are $1,300 \pm 66$ and $4,900 \pm 1030$ (WT), 140 ± 13 and 310 ± 40 (YZS84), 4.8 ± 1.2 and 19 ± 3.5 (YDP19), and 3.3 ± 0.54 and 97 ± 14 (YDP135). The indicated units of β -galactosidase activity represent the means for three independent transformants assayed in duplicate; standard deviations are indicated by error bars.

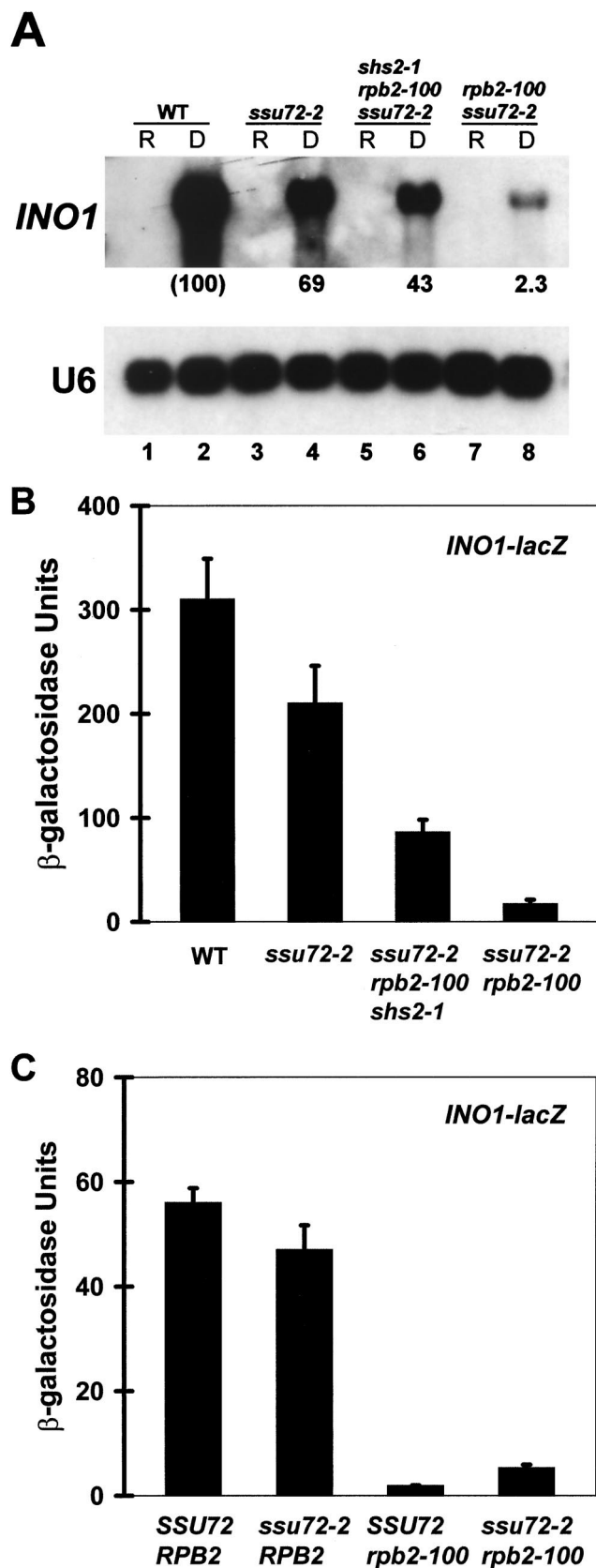


FIG. 5. Effects of *ssu72* and *shs* mutations on *INO1* expression. (A) The top panel indicates *INO1* transcript levels, determined by Northern blot analysis, for strains grown under repressing (+Ino medium) (lanes R) and derepressing

activated expression, the measurable levels of β -galactosidase activity from the *INO1-lacZ* reporter under repressing conditions demonstrate that the effects of *ssu72-2* and *rpb2-100* on *INO1* expression can be accounted for by effects on noninduced transcription.

The *INO1-lacZ* reporter assays were repeated using strain YDP135 that had been complemented with plasmid-borne copies of *SSU72* and/or *RPB2*, allowing the effects of *ssu72-2* and *rpb2-100* to be assessed in an isogenic background independent of *shs2-1*. Consistent with the previous results, *ssu72-2* alone had only a minor effect on *INO1-lacZ* expression, whereas the effects of the *rpb2-100* single and *ssu72-2 rpb2-100* double mutations were more dramatic, yielding less than 10% of normal activity (Fig. 5C). Thus, *ssu72-2* exerts adverse effects on gene expression that are markedly enhanced by the *rpb2-100* allele, and these effects are manifest at the level of noninduced transcription.

The effects of *ssu72-2* and *rpb2-100* were also assessed using the *PGK1* promoter, which is expressed at a high constitutive level. A *PGK1-lacZ* reporter plasmid was introduced into each strain, and β -galactosidase activities were measured (Fig. 6). Expression from the *PGK1* promoter was diminished to 60% of normal in the *ssu72-2* mutant and to 40% of normal in the *ssu72-2 rpb2-100 shs2-1* triple mutant. Expression was markedly diminished in the *ssu72-2 rpb2-100* double mutant, to 3% of normal. Although these experiments do not distinguish non-induced from activated expression, the dramatic effect of the double mutant indicates that *ssu72-2* and *rpb2-100* can exert synergistic effects on gene expression. The more adverse effect of *rpb2-100* in the *SHS2*⁺ background is consistent with its effect on *INO1* expression (Fig. 5). These results demonstrate that *shs2-1*, which is required in addition to *rpb2-100* for suppression of the *ssu72-2* Ts⁻ phenotype (Fig. 2), also suppresses the adverse effects of *rpb2-100* on noninduced transcription.

Physical interaction between Ssu72 and RNAP II. The genetic interactions among *ssu72*, *sua7*, and *rpb2* suggest that Ssu72 interacts directly with the core transcriptional machinery. Indeed, Ssu72 directly binds TFIIB, consistent with allele-specific interactions between *ssu72* and *sua7* (54). Physical interaction between Ssu72 and RNAP II was investigated by a coimmunoprecipitation assay using purified yeast RNAP II and a monoclonal antibody directed against the Rpb1 subunit of RNAP II. Input proteins were ³⁵S-labeled Ssu72, with labeled TBP and TFIIB as controls. RNAP II is known to bind TFIIB, but not TBP, whose interaction with RNAP II requires TFIIB as a bridging factor. The Ssu72 input included two forms of the protein, corresponding to full-length Ssu72 and N-terminally truncated Ssu72 resulting from initiation at AUG codon 23. The results demonstrate that RNAP II binds stably to TFIIB and Ssu72 but not to the TBP control (Fig. 7A). Thus, the genetic interaction between *ssu72-2* and *rpb2-100* is likely to reflect a physical interaction between Ssu72 and RNAP II.

The N terminus of Ssu72 is essential for Ssu72 function and cell viability, yet RNAP II binds the smaller form of Ssu72, lacking the N-terminal 22 residues. This was further investi-

(-Ino medium) (lanes D) conditions. Strains are identical to those in Fig. 4. The bottom panel is a loading control showing U6 RNA levels. (B) β -Galactosidase activities expressed from the *INO1-lacZ* plasmid pJS325 under repressing conditions. Activity units are 310 ± 39 (wild type [WT]), 210 ± 36 (YZS84), 86 ± 12 (YDP19), and 17 ± 4.1 (YDP135). Error bars indicate standard deviations. (C) Same as panel B, except the host strain in each case is YDP135 (*ssu72-2 rpb2-100*) carrying the *INO1-lacZ* reporter and plasmid-borne copies of wild-type *SSU72*, *RPB2*, or vectors alone, as indicated by the genotypes. Activity units are 56 ± 2.8 (WT), 47 ± 4.7 (*ssu72-2*), 1.9 ± 0.07 (*rpb2-100*), and 5.3 ± 0.63 (*ssu72-2 rpb2-100*).

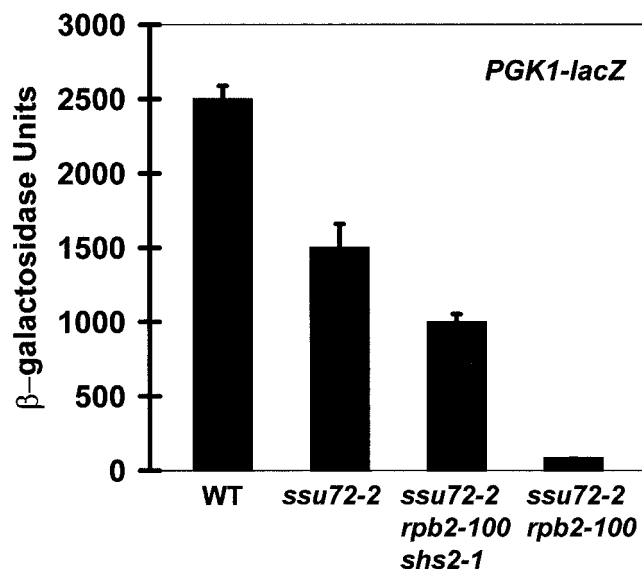


FIG. 6. β -Galactosidase activities expressed from a *PGK1-lacZ* plasmid. Strains are identical to those in Fig. 4 and in Fig. 5A and B, except that each was transformed with the *PGK1-lacZ* reporter plasmid pN1086. Activity units are 2,500 \pm 88 (wild type [WT]), 1,500 \pm 160 (YZS84), 1,000 \pm 52 (YDP19), and 86 \pm 3.6 (YDP135).

gated by asking whether specific mutations in the Ssu72 N terminus affect RNAP II binding. The *ssu72-1* allele, which enhances the *sua7-1* mutation, encodes a 10-amino-acid duplication in the N terminus of Ssu72 (residues 9 to 18), and *ssu72-4* encodes a serine replacement of cysteine 15 that abolishes cell viability (48). The interaction between Ssu72 and RNAP II appeared to be unaffected by the *ssu72-2* (R129A) and *ssu72-4* (C15S) mutations (Fig. 7B, lanes 5 to 8). Interestingly, however, the 10-amino-acid duplication resulted in preferential binding of RNAP II to the longer form of Ssu72 (Fig. 7B, lanes 1 to 4). These results confirm that Ssu72 binds directly to RNAP II and imply that the Ssu72 N terminus affects this interaction.

DISCUSSION

The results in this paper define genetic and physical interactions between Ssu72 and RNAP II and support a role for Ssu72 in basal (noninduced) transcription by RNAP II. Ssu72 was initially identified based on a genetic interaction with TFIIB. The *ssu72-1* allele confers a synthetic T_s^- growth defect that is dependent upon the *sua7-1* mutation (48). The interaction of *ssu72-1* with *sua7* is specific, resulting in synthetic phenotypes only in combination with *sua7* alleles that affect transcription start site selection (54). Ssu72 is also genetically linked to Sub1 (Tsp1), a homolog of the human general transcriptional cofactor PC4, in that a *sub1* deletion also enhances *sua7* mutations and does so in an allele-specific manner that is identical to the specificity of the *ssu72-1 sua7* interactions (54). Consistent with the genetic data, TFIIB directly interacts with the Ssu72 and Sub1 proteins (27, 54). Here we demonstrate that Ssu72 binds stably to RNAP II and that the *ssu72-2* and *rpb2-100* alleles affect basal transcription. Taken together, these data demonstrate functional interactions of Ssu72 with TFIIB, Sub1, and Rpb2 and provide strong support for Ssu72 as an RNAP II transcription factor.

We do not know the specific function of Ssu72. Our efforts to characterize Ssu72 have been hampered because sequence

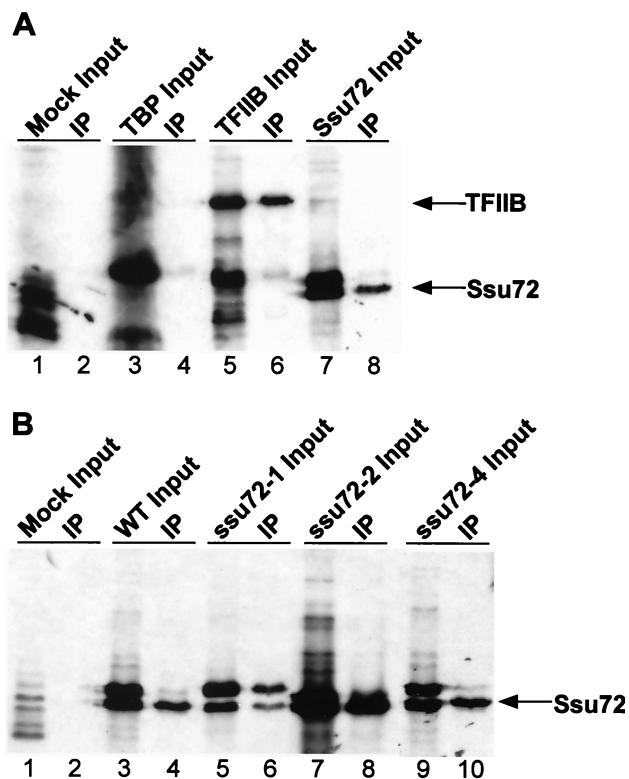


FIG. 7. Physical interaction between Ssu72 and RNAP II. (A) Equal amounts of highly purified yeast RNAP II were incubated with 35 S-labeled mock, TBP, TFIIB, and Ssu72 input proteins. Samples were immunoprecipitated (IP) using a monoclonal antibody directed against the C-terminal repeat domain of Rpb1. Following centrifugation, samples were washed three times with buffer containing 100 mM potassium acetate plus 0.1% NP-40 and once with buffer containing 100 mM potassium acetate and then resuspended in sample buffer. Input proteins (5% of the total; lanes 1, 3, 5, and 7, respectively) and the precipitates (50% of the total; lanes 2, 4, 6, and 8, respectively) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by autoradiography. WT, wild type. (B) Same as panel A, except that different forms of Ssu72 were used as input proteins. The *ssu72-1* allele encodes a duplication of amino acid residues 9 to 18, *ssu72-2* encodes the R129A replacement, and *ssu72-4* encodes an inviable C15S replacement. The upper and lower bands of Ssu72 input proteins correspond, respectively, to the full-length protein and a truncated protein whose N terminus corresponds to methionine at position 23. In the case of Ssu72-1, RNAP II interacts with both the full-length and truncated forms of the protein. Control reactions lacking RNAP II failed to coimmunoprecipitate Ssu72 (data not shown).

tags added to the N terminus appear to expose a cryptic proteolytic site just within the Ssu72 N terminus, thereby removing the tag, whereas sequences added to the C terminus render Ssu72 nonfunctional in vivo, perhaps due to the conserved, tandem aromatic residues at the C terminus (Fig. 1). Interestingly, there are no apparent Ssu72 homologs in bacterial or archaeal genomes, implying that Ssu72 function is specific to eukaryotes. In yeast, depletion of Ssu72 causes growth arrest and diminishes total poly(A) RNA levels by about 20 to 40% but is without effect on RNAP I and RNAP III transcription (Pappas, unpublished results). These results suggest that Ssu72 is critical for transcription of many, but not all, RNAP II genes yet plays no role in RNAP I or RNAP III transcription.

All evidence points to a role for Ssu72 in core promoter function. The original *ssu72-1* allele, which encodes a 10-amino-acid duplication near the N terminus, affects transcription start site selection (48). This effect is dramatic, but it is dependent upon a *sua7* allele (TFIIB) that also affects initiation. Here we demonstrate that the *ssu72-2* allele adversely affects

noninduced transcription. The *rpb2-100* suppressor of *ssu72-2* also diminishes noninduced transcription, and this effect can be synergistic with *ssu72-2*. The magnitude of the *ssu72-2* effect on transcription appears to be promoter specific: whereas *CYC1* transcription is diminished nearly 10-fold, less than 2-fold effects are observed for *INO1* and *PGK1* expression. The promoter elements responsible for these effects are unknown but can readily be addressed by whole-genome microarray analysis using wild-type and *ssu72-2* strains.

It is noteworthy that *rpb2-100* was isolated as a suppressor of the *ssu72-2* Ts^- growth defect, yet *rpb2-100* and *ssu72-2* exert similar effects on transcription. This anomaly can be explained by the results from genetic analysis of the YDP19 revertant, revealing that mutations in two genes, *rpb2-100* and *shs2-1*, are required for suppression of the *ssu72-2* Ts^- phenotype. Indeed, the effect of either allele in the absence of the other is to enhance the *ssu72-2* phenotype, essentially eliminating growth at 37°C (Fig. 2). This result not only accounts for the similar effects of *ssu72-2* and *rpb2-100* on transcription but also is consistent with their synergistic effects on transcription from the *PGK1* promoter (Fig. 6). Although isolated as a suppressor, the *rpb2-100* allele is an enhancer of *ssu72-2* in the absence of *shs2-1*.

The *rpb2-100* allele encodes a single amino acid replacement, R512C, located at the first position within homology block D of Rpb2 (Fig. 3). The viability of the R512C mutant indicates that R512, despite its phylogenetic invariance, is not functionally invariant. Not only does the *rpb2-100* allele (in combination with *shs2-1*) suppress the *ssu72-2* Ts^- phenotype, but the *ssu72-2 rpb2-100 shs2-1* strain (YDP19) exhibits only marginally impaired growth on rich (YPD) medium at 30°C. The viability of *rpb2-100* mutants cannot be attributed to a suppressive effect of *ssu72-2* on *rpb2-100*, because an *rpb2-100* mutant is also viable in an *SSU72⁺* background, with a growth rate comparable to that of YDP19 (Pappas, unpublished results). Furthermore, the *rpb2-100 SSU72⁺* strain exhibits the same severe Cs^- and Ino^- phenotypes as YDP19, thereby confirming that these phenotypes are due to the R512C replacement.

Other *rpb2* alleles have been isolated in genetic selections for mutations that affect core promoter function (3, 4, 14, 19). The *spt* alleles of *RPB2* were identified as suppressors of δ -element insertions at the *HIS4* and *LYS2* promoters (19). These alleles encode amino acid replacements in the region between homology blocks B and C of Rpb2 and underscore a role for the Rpb2 subunit in core promoter recognition. The *sit1* and *sit2* alleles of *RPB1* and *RPB2*, respectively, were isolated as enhancers of *HIS4* transcription in the absence of the *trans* activators Gcn4, Bas1, and Bas2 (4). These mutations are comparable to *rpb2-100* in that they also affect noninduced transcription, albeit with opposite effects. Furthermore, the *sit1* and *sit2* mutants, like the *rpb2-100* mutant, are inositol auxotrophs. The *sit1* alleles encode Rpb1 amino acid replacements in either region D or region F, both of which are within or near the active center of RNAP (3). In the *Thermus aquaticus* RNAP structure, the *sit1* region F replacements are adjacent to R428, the counterpart of R512 in Rpb2. The opposite effects of the *sit1* and *rpb2-100* mutations on transcription might be accounted for by their proximity to the secondary channel, where the distinction between the stimulatory or inhibitory effects might be accounted for by structural changes that affect substrate access to the active site and, consequently, the efficiency of promoter clearance.

A crystal structure of yeast RNAP II was recently solved at a resolution of 3 Å (10). Unfortunately, R512 is located within an approximately 13-residue gap that precludes interpretation

of the R512C replacement in the context of the RNAP II three-dimensional structure. However, a crystallographic structure of RNAP from *E. coli* has been determined at a 12-Å resolution (11, 12), and, more recently, that of RNAP from *T. aquaticus* has been determined at a 3.3-Å resolution (56). R512 is highly conserved, corresponding to R548 and R428 of the β subunits of RNAPs from *E. coli* and *T. aquaticus*, respectively (Fig. 3). The sequence conservation among all forms of RNAP and the nearly superimposable structures of *E. coli* and *T. aquaticus* RNAPs (32) allow for the R512C replacement to be interpreted in the context of the *T. aquaticus* crystal structure. Moreover, a comprehensive description of site-specific interactions between catalytically competent RNAP and promoter open DNA was recently defined by protein-DNA photo-cross-linking (31). Accordingly, R428 forms part of the binding site for double-stranded DNA downstream of position +1 and is positioned to make direct contact with the phosphate 5' to position +6 of the nontemplate strand. The position of this residue suggests possible roles for R428 in stabilizing RNAP-DNA interaction, in facilitating clamping of RNAP on duplex DNA, in facilitating RNAP translocation, and/or in defining the downstream boundary of the transcription bubble. Furthermore, the catabolite gene activator protein has no effect on cross-linking between RNAP and the core promoter region (31). These results readily account for the effects of *rpb2-100* on noninduced transcription and underscore our premise that Ssu72 is a core RNAP II transcription factor.

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