Stimulation of Transcription by Mutations Affecting Conserved Regions of RNA Polymerase II

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Mutations that increase the low-level transcription of the *Saccharomyces cerevisiae* HIS4 gene, which results from deletion of the genes encoding transcription factors BAS1, BAS2, and GCN4, were isolated previously in *SIT1* (also known as *RPO21*, *RPB1*, and *SUA8*), the gene encoding the largest subunit of RNA polymerase II (RNAPII). Here we show that *sit1* substitutions cluster in two conserved regions of the enzyme which form part of the active site. Six *sit1* mutations, affect region F, a region that is involved in transcriptional elongation and in resistance to α-aminatin. Four *sit1* substitutions lie in another region involved in transcriptional elongation, region D, which binds Mg^{2+} ions essential for RNA catalysis. One region D substitution is lethal unless suppressed by a substitution in region G and interacts genetically with *PPR2*, the gene encoding transcription elongation factor IIS. Some *sit1* substitutions affect the selection of transcriptional start sites at the *CYC1* promoter in a manner reminiscent of that of *sua8* (*sua* stands for suppression of upstream ATG) mutations. Together with previous findings which indicate that regions D and G are in close proximity to the 3′ end of the nascent transcript and that region F is involved in the translocation process, our results suggest that transcriptional activation by the *sit1* mutations results from alteration of the RNAPII active center.

The structures and functions of eukaryotic RNA polymerases I, II, and III are well conserved during evolution (reviewed in references 6, 41, and 53). Structurally, these three enzymes contain two large subunits which are homologous to the two largest subunits, β and β′, of *Escherichia coli* RNA polymerase (RNAP) and which contain the active site of the enzyme (reviewed in references 6, 41, and 53). In addition to containing the two largest subunits, eukaryotic RNAPs also contain approximately 10 smaller subunits, some of which are present in both RNAPI and RNAPII or in all three enzymes (reviewed in references 41 and 53).

Mutations affecting the two largest subunits of *Saccharomyces cerevisiae* RNAPII were isolated by Arndt et al. (8) in a genetic selection for *sit* mutations (*sit* stands for suppressor of initiation of transcription defect) that can increase transcription of the *HIS4* gene in a strain in which the *BAS1*, *BAS2*, and *GCN4* genes were deleted. The last three of these genes encode transcription factors which activate transcription of *HIS4* through specific DNA elements located in the promoter region of the gene (8). A yeast strain which lacks BAS1, BAS2, and GCN4 is auxotrophic for histidine because *HIS4* is transcribed poorly (7, 8). Transcription of *HIS4* can be increased by mutations in *SIT1* (also known as *RPO21*, *RPB1*, and *SUA8*) and *SIT2* (also known as *RPO22* and *RPB2*), which encode the largest and second-largest subunits of RNAPII, respectively. Transcription of *HIS4* in *sit1* and *sit2* mutant strains, as in a wild-type strain, requires the presence of a functional TATA element as well as a binding site for RAP1 in the promoter. The requirement for these two promoter elements most likely reflects a need for RAP1 to antagonize the repressing effect of chromatin and allow the general transcription machinery to gain access to the *HIS4* promoter, at least in part through an interaction between TBP and the TATA box (22). RAP1 binds to the *HIS4* promoter but does not efficiently stimulate its transcription in the absence of BAS1, BAS2, and GCN4 (8, 22).

One can envisage two general mechanisms by which the *sit1* and *sit2* mutations affect RNAPII in order to increase synthesis of *HIS4* mRNAs. First, these mutations could increase the number of initiation complexes that assemble at or are recruited to the *HIS4* TATA box. Results of experiments performed in vivo in which high levels of transcription were obtained by artificially tethering the RNAPII holoenzyme to promoter DNA suggested that recruitment of the holoenzyme to the promoter is a rate-limiting step in transcription (9).

Second, the *sit1* and *sit2* mutations could affect other steps that can be rate limiting following transcription initiation, such as an early block to elongation or premature termination (14, 28, 36, 39, 52). In this case, the *sit1* mutations could increase the number of RNAPII molecules that reach the end of the transcription unit, without affecting the overall rate of initiation, by increasing the processivity of RNAPII during elongation. The increased processivity of the enzyme could affect either early elongation, by increasing the frequency at which the initiation complexes leave the *HIS4* promoter and enter the elongation mode (promoter clearance), or later stages of elongation, by decreasing the rate of premature termination. As a first step toward understanding how the *sit1* mutations affect transcriptional activation, we have identified regions of the largest subunit of RNAPII that are affected by these mutations.

MATERIALS AND METHODS

S. cerevisiae **strains and yeast manipulations.** All *sit1* strains used in this study were isolated as spontaneous His⁺ revertants of strain L3110 (*MAT**a** gcn4-2 bas1-2 bas2-2 ura3-52*) as previously described (7, 8). The yeast strain YF2047 (*MAT*α *gcn4-2 bas1-2 bas2-2 rpo21::LEU2 leu2 trp1::hisG ura3-52* [pJS121; *RPO21* on *URA3 CEN/ARS* plasmid]) was constructed in several steps. First, the *TRP1* locus of L3110 was changed to *trp1::hisG* as previously described (1) to

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create yeast strain YF2037. YF2037 was then mated with yeast strain CY843 (*MATα gcn4-2 bas1-2 bas2-2 ura3-52 leu2*). The resulting diploid was sporulated, and tetrads were dissected. A Leu⁻ Trp⁻ haploid progeny, YF2044 (*MATα gcn4-2 bas1-2 bas2-2 ura3-52 trp1::hisG leu2*), was then transformed to uracil prototrophy with plasmid pJS121 (*RPO21 URA3 CEN/ARS*) (4). The *RPO21* locus of this merodiploid strain was then disrupted (*rpo21::LEU2*) in one step to create strain YF2047. The yeast strain YDW383, which carries the *sua8-1* mutation as well as an isogenic *SUA8* wild-type strain, T16, have been described previously (13). YF2277 (*MATα rpo21::ADE2* [pJS121]), a strain congenic with W303-1B (*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1-100 ssd1-d*) contains a disruption of the chromosomal *RPO21* allele caused by a replacement by *ADE2* of the *RPO21* sequence from positions −718 to +660 (the A of the translation initiation codon is +1). YF2278 (*MATα rpo21::ADE2 ppr2::hisG* [pJS121]) is isogenic to YF2277 except for the disruption of *PPR2* by *hisG*. This was introduced by using plasmid pJD3, which contains *ppr2::hisG-URA3-hisG* (a gift of C. M. Kane, University of California at Berkeley). YF2277 and YF2278 were generated by standard genetic techniques. Details of their construction are available upon request. Growth media used were as described previously (45). Yeast transformation was performed as described previously (26).

Plasmids. Plasmid pJAY61, which was used for the isolation of *sit1* alleles, was constructed in two steps. First, an integrating plasmid carrying *URA3* as a selectable marker and the wild-type *SIT1* gene on a *Hin*dIII-*Eco*RI genomic DNA fragment (the *Hin*dIII and *Eco*RI sites are located 1.6 kb upstream of the *SIT1* translation initiation codon and 1.7 kb downstream of the translation termination codon, respectively) was constructed. Second, a deletion was introduced in the *SIT1* open reading frame by removing the DNA sequences contained between an *Avr*II site (located 162 nucleotides downstream of the translation initiation codon) and a *Sna*B1 site (located 16 nucleotides downstream of the translation termination site) and replacing them with a *Bam*HI linker (5′-GGGATCCC-3′) after filling in the protruding ends of the *Avr*II site with the Klenow fragment of DNA polymerase I. Plasmid pJA483 was created by subcloning a 5.7-kb *Eco*RI-*Hin*dIII fragment encompassing *RPO21* into the *Eco*RI and *Hin*dIII sites of pFL39 (*TRP1 CEN6 ARS*) (15). Plasmids pM50 and pM107, which carry *CYC1-lacZ* and *cyc1-5000-lacZ*, respectively, cloned in a single-copy (*URA3*) plasmid, have been described previously (38).

DNA manipulation and sequencing. All DNA manipulations were performed essentially as described previously (30). DNA sequencing of plasmid DNA was performed by the chain termination method (40) using primers synthesized on the basis of the *SIT1* sequence.

PCR amplification of the DNA sequence encoding conserved region F. The DNA sequence between nucleotides 2155 and 2907 (in the numbering system of Allison et al. [3]), which encodes the conserved region F of RPO21, was amplified by PCR from genomic DNA of several *sit1* alleles by using the following two oligonucleotides: 5′-CGGGATCCGGTGTAGTAGAGAAAAAAC-3′ and 5′-CGGGATCCTGAATAACGTTACCCAATG-3′. The resulting amplified fragments containing *Bam*HI sites at both ends (generated by the two primers) were then cut with *Bam*HI and cloned into the *Bam*HI site of plasmid YEp24 (17). For each *sit1* allele amplified and cloned, two independently isolated plasmids were sequenced. The amplified region F DNAs were also cut with *Ppu*MI and *Xba*I and subcloned between the *Ppu*MI and *Xba*I sites of pJA483 (*RPO21 TRP1 CEN6 ARS*).

PCR amplification of the DNA sequence encoding regions B, C, D, and E. The DNA sequence between the *Spe*I and *Ppu*MI sites of *RPO21*, which encodes conserved regions B, C, D, and E, was amplified by PCR using the following two oligonucleotides: 5′-AGAGCGAAAATTGGTGGTC-3′ and 5′-GCCTCTGCA ATTGTCTCTG-3′. The amplified fragment was cut with *Spe*I and *Ppu*MI and was subcloned into pJA483 (*RPO21 TRP1 CEN6 ARS*) cut with *Spe*I and *Ppu*MI. For each *sit1* allele, the DNA sequence between *Spe*I and *Ppu*MI was determined in two independently isolated plasmids.

β-Galactosidase assays. β-Galactosidase activity was measured as described by Miller (34). Cells were grown to an optical density (at 600 nm) of 0.6 in SC-Ura medium (45) with glucose as a carbon source. For each measurement, β-galactosidase activity was determined on three independent cultures.

RESULTS

Molecular cloning of *sit1* alleles. As a first step toward determining the mechanism by which mutations which affect RNAPII can increase transcription of *HIS4* in the absence of the BAS1, BAS2, and GCN4 proteins, we set out to identify which regions of the largest subunits are altered by the *sit1* mutations. A gap repair strategy (37) was used to clone five independent *sit1* alleles (*sit1-4, -5, -7, -8,* and *-9*) as well as the wild-type *SIT1* allele from the parental yeast strain, L3110 (7). In order to localize the *sit1* mutations, two independently cloned copies of each *sit1* allele were digested with several restriction endonucleases, each of which recognizes a different 4-bp site (*Alu*I, *Hae*III, *Taq*I, and *Sau*3A), in the hope that

TABLE 1. Nucleotide and amino acid sequence changes in *sit1* alleles

Region and allele	Nucleotide change[a]	Amino acid substitution
Region F		
sit1-7	G 2501 to A	Gly 730 to Asp
sit1-5	G 2587 to C	Ala 759 to Pro
sit1-290	G 2603 to T	Cys 764 to Phe
sit1-290	G 2314 to A	Asp 668 to Asn
sit1-9	G 2771 to A	Gly 820 to Asp
sit1-4	C 2807 to T	Ala 832 to Val
sit1-246	C 2807 to T	Ala 832 to Val
Region D		
sit1-8D	A 1747 to T	Asn 479 to Tyr
sit1-252	A 1771 to T	Met 487 to Leu
sit1-261	A 1771 to T	Met 487 to Leu
sit1-278	A 1646 to C	Asn 445 to Thr
Region G		
sit1-8G	C 3539 to T	Ala 1076 to Val

[a] The positions of the nucleotide changes are given in the numbering system of Allison et al. (3).

certain *sit1* mutations would either create or destroy a site so as to give rise to a restriction fragment length polymorphism. A *Sau*3A restriction fragment was detected in the *sit1-7* allele that was not present in any of the other mutant alleles or in the wild-type gene (data not shown). This *Sau*3A polymorphic fragment was cloned and sequenced. The mutation creating the new *Sau*3A site results in the replacement of glycine 730 with an aspartic acid residue in the conserved region F of the largest subunit of RNAPII (Table 1), one of eight regions of this polypeptide that have been conserved during evolution (27).

Six *sit1* alleles carry mutations affecting conserved region F of *RPO21*. Additional *sit1* mutants were then examined for the presence of mutations affecting region F (nucleotides 2200 to 2900 in the numbering system of Allison et al. [3]). When the other cloned *sit1* alleles (*sit1-4, -5, -8,* and *-9*) were examined, region F mutations were found in *sit1-4, sit1-5,* and *sit1-9* (Table 1 and Fig. 1). In the case of *sit1* mutants for which the *sit1* allele was not cloned, the DNA sequence encoding region F (between nucleotides 2200 and 2900) was amplified from genomic DNA by PCR and cloned into a plasmid and two independent plasmid isolates were sequenced. Of five *sit1* mutants analyzed in this fashion (*sit1-290, -246, -252, -261,* and *-278*), two (*sit1-290* and *-246*) were found to carry mutations that affect region F (Table 1 and Fig. 1). One of them (*sit1-290*) had two mutations, one in region F and one just upstream of region F (Table 1). Since four of the *sit1* alleles tested (*sit1-8, -252, -261,* and *-278*) did not carry a mutation affecting region F, at least one other region of *SIT1* can be altered so as to confer the Sit phenotype (see below).

In order to determine whether the region F mutations that were identified are both necessary and sufficient to cause the Sit phenotype, the DNA fragments encoding each mutant region F (nucleotides 2224 to 2844 [*Ppu*MI-*Xba*I fragment]) were subcloned individually into an otherwise wild-type *SIT1* gene (*sit1-246* was not used for this analysis since it carries the same mutation as *sit1-4*). The reconstructed *sit1* mutant alleles were tested for the ability to support growth on medium lacking histidine (the Sit phenotype) by two different assays. In the first assay, which relies on the ability of the *sit1* alleles to complement a disrupted allele of *SIT1* (*sit1::LEU2*), recon-

Region F

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sit1                                    D(7)                          P(5) F(290)
S. cerevisiae   GMTLRESFEDNVVRFLNEARDKAGRLAEVNLKDLNNVKQMVMAGSKGSFINIAQMSACVGQQSVEGKRIA   776
S. pombe        .........AK.S.I..Q...N...S..HS...S.......A..........S..........I......P   782
A. thaliana     .R.M.DT..NR.DQV..K...D..SS.QKS.AET..L.A..T..........S..T......N......P   795
C. elegans      .N...QT..NK.NQI..D...RT.SS.QKS.SEF..F.S..VS.....K...S.VI......N......P   789
D. melano.      .N...QT..NK.N.I..D....T.GS.KKS.TEY..L.A..VS.....N...S.VI......N......P   791
M. musculus     .N...QT..NQ.N.I..D....T.SS.QKS.SEY..F.S..VS.A...K...S.VI.V....N......P   879
S. c. RNAPIII   .CNEEQTL.AKIGGL.SKV.EEV.DVCINE.DNW.APLI.ATC.....TL.VS..V.V....IIS.N.VP   824
S. acidocal.(A) .R.IE..L.SYILDT.DKL.KV..EI.T.Y.DPF...YI.AIT.AR..EL..T..T.LL.....R.E..R   758
E. coli         WAAAANDRVSKAMMDN.QTETVINRDGQ.EKQVSF.SIYM.ADS.AR..AAQ.R.LAGMR.LMAKPDGS.-   754
E. coli mut.                                I  FDT F       H       D I
Ama C. elegans                                                    Y         E
Ama D. melano.                      H
Ama M. musculus              F    P                              F             D

sit1                                    D(9)          V(4, 246)
S. cerevisiae   FGFVDRTLPHFSKDDYSPESKGFVENSYLRGLTPQEFFFHAMGGREGLIDTAVKTAETGYIQRRLVKALE   846
S. pombe        ...KY......P....D....R..I................A..........................M.   852
A. thaliana     ...DG......T....G...R............................S...........M.        860
C. elegans      ...RH......I....G........A....S..........................I..M.         859
D. melano.      Y..RK......I........R........A...S..Y....................I..M.         861
M. musculus     ...KH......I........R........A....T......................I.SM.         869
S. c. RNAPIII   D..Q..S.....P.NSKT.Q.....R..FFS..S.P..L...IS.....V...........MS...M.S..  894
S. acidocal.(A) R.YRE...SL.KYG.IA..AR...K..FM...S.Y.M....A.V.....SQS..M....IN..S       828
E. coli         -------------------IETPITANFRE..NVLQY.ISTH.A.K..A...L...NS..LT....DVAQ   805
E. coli mut.                               K       K             H       T   V    C
E. coli St1                                                               F
B. sub. St1                                                             G
RNAPIII C160-270        AA
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Region D

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rpb1-17                                              I
sua8           S(1,2)
sit1           T(278)                          Y(8D)    L(252,261)
S. cerevisiae   VLFNRQPSLHKMSMMAHRVKVIPYSTFRLNLSVTSPYNADFDGDEMNLHVPQSEETRAEL  501
S. pombe        .I.............G..IR.M.........................M.........I   507
A. thaliana     ..............I.G..IRIM.......................M.....F.....V   511
C. elegans      IV.....T.......G....IL.W...M...................L...L.....I   505
D. melano.      .I.....T.......G....L.W...M...C.................V...V     507
M. musculus     .I.....T.......G...RIL.W......T..............L...L.....I   514
S. acidocal.(A) .........RI.....K.R.L.GR......LDCP..................AI..A   476
E. coli         ..L..A.T..RLGIQ.FEPVL.EGKAIQ.HPL.CAA........Q.AV...LTL.AQL.A   480
S. c. RNAPIII   ..........RL.ILS.YA..IR.WR.....EC.CT.................T..A...A   531
RNAPIII C160-112              I   Y
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Region G

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rpb1                           I(502)         F(501)
sit1                          V(8G)
S. cerevisiae   GEMVGVLAAQSIGEPATQMTLNTFHFAGVASKKVTSGVPRLK   1102
S. pombe        .....T....................Y...S..N..L......   1105
A. thaliana     ...I.CV..................Y...SA.N..L.....R   1117
C. elegans      .....A.....L.............Y...SA.N..L......   1125
D. melano.      .....A.....L................S..N..L......   1116
M. musculus     .....A.....L.............Y...SA.N..L......   1125
S. acidocal.(C) ..AI..VT...V...G.....R......IRELN..L.L...I    106
S. c. RNAPIII   .TAI.AIG.......G.....K.........MN..L....I.   1127
E. coli         ..AI..I........G..L.MR...IG.A..RAAAESSIQV.    953
E. coli mut.                  CMY
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FIG. 1. Locations of *sit1* amino acid substitutions. The amino acid sequences of regions F, D, and G of *S. cerevisiae* RNAPII (*S. cerevisiae*) are compared to those of other eukaryotes: *Schizosaccharomyces pombe* RNAPII (*S. pombe*), *Arabidopsis thaliana* RNAPII (*A. thaliana*), *Caenorhabditis elegans* RNAPII (*C. elegans*), *D. melanogaster* RNAPII (*D. melano.*), *Mus musculus* RNAPII (*M. musculus*), *Sulfolobus acidocaldarius* (*S. acidocal.*) subunit A or C as indicated, *E. coli* RNAP, and *S. cerevisiae* RNAPIII (*S. c.* RNAPIII). Residues identical to those of *S. cerevisiae* RNAPII are indicated by dots; gaps are indicated by hyphens. For each *sit1* mutation, the substituted amino acid is indicated above the wild-type amino acid and the designation of the allele is given in parentheses. A second substitution in the *sit1-290* polypeptide that is located upstream of region F is not shown (Table 1). Amino acid substitutions that confer resistance to the transcriptional inhibitor α-amanitin (Ama) in *C. elegans* (19), *D. melanogaster* (19), and *M. musculus* (10, 11) are also indicated. Single amino acid substitutions that affect elongation and termination by *E. coli* RNAP (49) are written below the sequence of the largest subunit of *E. coli* RNAP (*E. coli* mut.). Substitutions that confer streptolydigin resistance in *E. coli* (*E. coli* St1) (44) and *Bacillus subtilis* (*B. sub.* St1) (51) are indicated. The double substitutions in the *S. cerevisiae* RNAPIII C160-270 mutant (RNAPIII *C160-270*) (47) and C160-112 mutant (RNAPIII *C160-112*) (23) are indicated. The invariant Mg^{2+} binding motif NADFDGD in region D is underlined. Also indicated are the locations of the conditional lethal *rpb1-17* substitution (43), the *sua8-1* and *sua8-2* substitutions (13), and the mutations (*rpb1-501* and *-502*) that confer an Spt phenotype (25).

structed *sit1* alleles carried on a single-copy plasmid were introduced into the diploid yeast strain YF2201 (*MATa/MATα sit1::LEU2/SIT1 gcn4-2/gcn4-2 bas1-2/bas1-2 bas2-2/bas2-2 ura3-52/ura3-52 leu2/leu2 trp1::hisG/trp1::hisG*). Following sporulation and tetrad dissection, haploid progeny that inherited the plasmid-borne reconstructed *sit1* allele and the chromosomal disrupted *SIT1* allele were tested for growth on medium lacking histidine. Reconstructed alleles *sit1-4, -5, -7, -9,*

and *-290* were capable both of supporting yeast cell growth and of bringing about the Sit phenotype (Fig. 2A).

The second assay that was used to test for the Sit phenotype is based on our observation that mutant *sit1* alleles (either reconstructed alleles or alleles derived entirely from a mutant strain), but not the wild-type *SIT1* allele, can confer a semidominant Sit phenotype in yeast strain YF2047 (*MATα gcn4-2 bas1-2 bas2-2 sit1::LEU2 ura3-52 leu2 trp1::hisG* [pJS121; *SIT1*
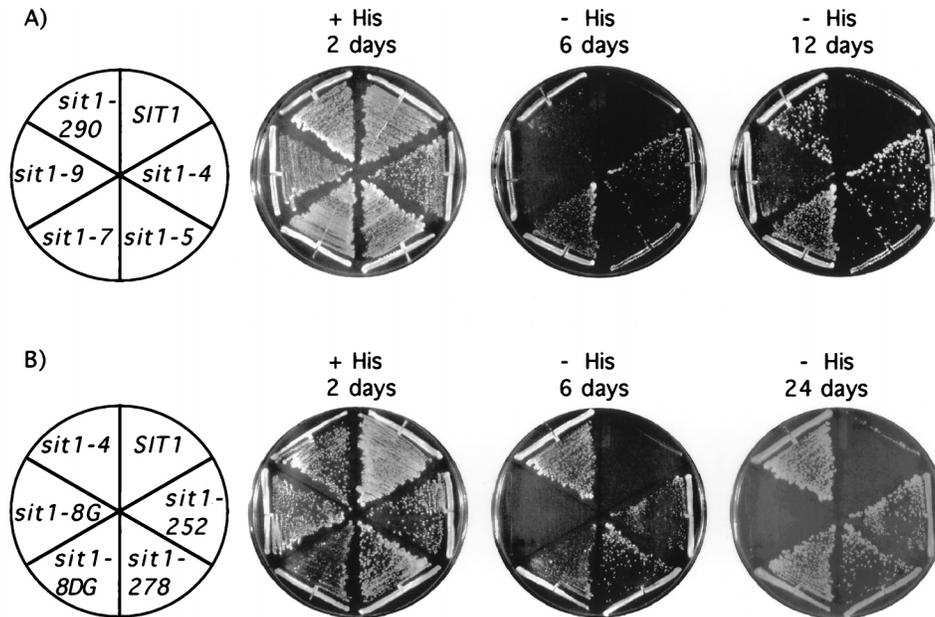
FIG. 2. Region F and D substitutions are necessary to confer the Sit phenotype. (A) Region F substitutions. Reconstructed *sit1* alleles carrying a region F mutation or the wild-type *SIT1* alleles were was assayed for the ability to confer the Sit phenotype by introduction into the diploid strain YF2201 (*MAT*a/*MAT*α *sit1*::*LEU2*/*SIT1 gcn4-2*/*gcn4-2 bas1-2*/*bas1-2 bas2-2*/*bas2-2 ura3-52*/*ura3-52 leu2*/*leu2 trp1*::*hisG*/*trp1*::*hisG*). The resulting transformants were then sporulated, 10 or more tetrads were dissected, and spores were allowed to germinate on YPD medium (45). Viable Trp+ (*sit1* allele on plasmid) Leu+ (*sit1*::*LEU2*) haploid progeny were then tested by streaking onto solid SD medium (45) containing (+ His) or lacking (− His) histidine. Cells were allowed to grow for the indicated number of days at 30°C. *sit1-9* required a longer incubation to show visible single colonies. (B) Region D substitutions. Reconstructed *sit1* alleles carrying region D mutations or the wild-type *SIT1* alleles were assayed for the ability to confer the Sit phenotype as described above. Cells were allowed to grow on solid SD medium (45) containing (+ His) or lacking (− His) histidine for the indicated number of days at 30°C. sit1-8G did not grow on medium lacking histidine, even when incubation was extended to 24 days. A region F substitution (*sit1-4*) is shown for comparison.

on *URA3* CEN/ARS plasmid]). This observation was surprising, because the Sit phenotype was shown previously to be recessive (8). It may be that the semidominant character of the Sit phenotype is influenced by the background of the strain. Alternatively, the degree of dominance of the Sit phenotype may depend on the relative level of expression of the mutant and wild-type alleles, which may be influenced by whether these alleles are expressed from their endogenous chromosomal location (8) or from episomal plasmids (this study). Regardless of the explanation, this assay was useful as an independent method of confirming that all five *sit1* mutations affecting region F (*sit1-4*, *-5*, *-7*, *-9*, and *-290*) are sufficient to confer a Sit phenotype (data not shown).

Mapping the *sit1* mutation in the *sit1-8* allele. The above analysis indicated that in some *sit1* alleles a region of the largest subunit other than region F is altered so as to bring about the Sit phenotype. Hybrid genes were constructed that contained various portions of *sit1-8* and wild-type *SIT1* (Fig. 3) in order to localize the mutation in the *sit1-8* allele, which does not contain a region F mutation (see above). These chimeric genes were tested by the two assays described above for the ability to support growth on medium lacking histidine. As shown in Fig. 3, the smallest region of *sit1-8* that was capable of conferring a His+ phenotype in an assay that relied on the semidominance of the Sit phenotype was located between nucleotides 971 and 2224 (hybrid g). The nucleotide sequence of this region was determined, and a mutation was found that replaces Asn 479 by Tyr in conserved region D of the largest subunit (Table 1 and Fig. 1). This mutation is referred to as *sit1-8D*. By the same assay, it was also noticed that yeast cells carrying a chimeric gene containing only the *sit1-8D* mutation (hybrid g) did not grow as well on medium lacking histidine as

did cells carrying the entire *sit1-8* allele (hybrid a). This enhancement of growth was associated with a second region of *sit1-8*, which was mapped to a region between nucleotides 3433 and 4621 (compare hybrid b with hybrid c and hybrid g with hybrid i). This region, however, was not sufficient by itself to bring about the Sit phenotype (hybrid h). The region located between nucleotides 3433 and 4621 was sequenced, and a mutation was found that results in the replacement of alanine 1076 by valine in conserved region G of the largest subunit (Table 1 and Fig. 1). The presence of both the *sit1-8D* and *sit1-8G* mutations was confirmed in other, independently isolated *sit1-8* alleles (data not shown).

Chimeric genes carrying both the *sit1-8D* and *sit1-8G* mutations or carrying either mutation alone were also tested for the ability to confer the Sit phenotype by the assay that relies on complementation of the *sit1*::*LEU2* allele. In this assay, hybrid g, which carries only the *sit1-8D* mutation, was unable to support growth of haploid cells even when histidine was present in the growth medium, indicating that the *sit1-8D* mutation is lethal. In contrast, hybrid i (*sit1-8DG*), which carries both the *sit1-8D* and *sit1-8G* mutations, was able to complement the *sit1*::*LEU2* allele and was also able to confer the Sit phenotype (Fig. 2B). Hybrid h, which carries only the *sit1-8G* mutation, was capable of complementing the *sit1*::*LEU2* allele but was unable to confer the Sit phenotype (Fig. 2B). Taken together these results suggest that the *sit1-8D* allele encodes a mutant subunit that assembles into an RNAPII complex which is capable of transcribing *HIS4* in the absence of BAS1, BAS2, and GCN4. However, this mutant RNAPII is not able to support cell growth unless it is modified further by the *sit1-8G* substitution, perhaps because genes that are either required or toxic for cell growth are aberrantly expressed (see Discussion).
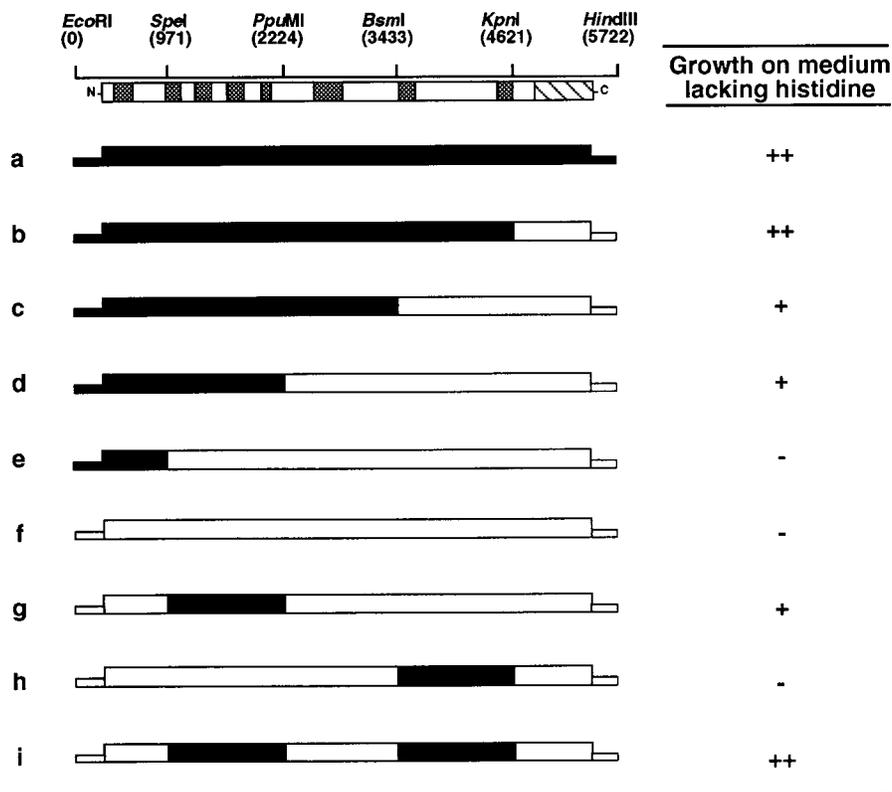
FIG. 3. Localization of mutations in the *sit1-8* allele. Schematic representation of the *SIT1* locus (*Eco*RI-*Hin*dIII fragment) showing the positions (in the numbering system of Allison et al. [3]) of various endonuclease sites that were used in the construction of the chimeric genes. The encoded SIT1 protein is diagrammed below the restriction map. The gray boxes represent regions (A to H) of the polypeptide that are most conserved evolutionarily (27), and the diagonally striped box represents the carboxy-terminal domain. The structures of the chimeric genes are indicated by open and filled boxes representing wild-type (*RPO21*) and mutant (*sit1-8*) sequences, respectively. Each hybrid gene is designated by a letter (a to i). The ability of each chimeric gene to confer a semidominant Sit phenotype was assayed by introducing it into yeast strain YF2047 (*MAT*α *gcn4-2 bas1-2, bas2-2 ura3-52 leu2 trp1::hisG sit1::LEU2* [pJS121; *SIT1* on *URA3 CEN/ARS* plasmid] [4]) and testing the resulting transformants for the ability to grow on solid SD medium (45) lacking histidine: ++, growth rate similar to that of a cell carrying the entire *sit1-8* allele; +, growth rate slower than that of a cell carrying the entire *sit1-8* allele; −, absence of growth.

In other experiments (see below), we found that the *sit1-8D* is not always lethal. This finding that the *sit1-8D* allele is capable of supporting cell growth in certain genetic backgrounds provides further evidence that this allele encodes a partially functional subunit. It also suggests that the pleiotropic phenotypes imposed by *sit1* mutations can vary according to the genetic background in which they are expressed (see Discussion).

Identification of other region D mutations. Other *sit1* mutant strains which did not carry a region F mutation (*sit1-252*, *-261*, and *-278*) were then tested for the presence of a region D mutation. For each of these mutants, a portion of *SIT1* encoding conserved regions B, C, D, and E (nucleotides 971 to 2224 [*Spe*I-*Ppu*MI fragment]) was amplified and subcloned into an otherwise wild-type *SIT1* gene. When tested for the Sit phenotype by the two assays described above, all three reconstructed *sit1* alleles were capable of supporting growth on medium lacking histidine. DNA sequencing revealed that two alleles, *sit1-252* and *sit1-261*, carry the same mutation which changes methionine 487 to leucine (Table 1 and Fig. 1). The mutation in the *sit1-278* allele replaces asparagine 445 by threonine (Table 1 and Fig. 1). The Sit phenotype imposed by region D substitutions is shown in Fig. 2B.

Effect of *sit1* mutations on start site selection. Amino acid substitutions located in and near conserved region D of the largest subunit were identified previously that suppress the

reduction in expression of *CYC1* brought about by the insertion of a nonfunctional AUG codon into the 5′ untranslated sequence of the gene (Sua phenotype [Sua stands for suppression of upstream ATG]) (38). Mutations in *SUA8* affect the position of the transcription start site at many yeast promoters in such a way that downstream start sites are favored relative to upstream ones (13). It was reported previously that some *sit1* mutations have a similar effect in that they increase the number of transcripts that start downstream of the *HIS4* translation initiation codon (8). This observation and the fact that some of the mutations characterized in this study change amino acids in region D which are also changed by *sua8* mutations (*sua8-1* and *sit1-278* change asparagine 445 to Ser and Thr, respectively) prompted us to determine whether *sit1* mutations can confer an alteration of start site selection at *CYC1*. Yeast strains carrying either the *sit1-8* (regions D and G), the *sit1-278* (region D), the *sit1-4* (region F), the *sit1-5* (region F), or the wild-type *SIT1* gene were transformed separately with a plasmid carrying a *CYC1-lacZ* reporter gene that either contains (*cyc1-5000* allele; plasmid pM107 [38]) or lacks (wild type *CYC1*; plasmid pM50 [38]) a nonfunctional ATG codon in the *CYC1* 5′ untranslated region. As controls, the same two plasmids were also introduced into a strain carrying the *sua8-1* mutation (YDW383 [13]) and an isogenic *SUA8* wild-type strain (T16 [13]). For each strain, the levels of β-galactosidase activity expressed from the *CYC1* (wild-type) allele or from the

TABLE 2. Effects of *sit1* mutations on expression of *CYC1* and *cyc1-5000*

Allele	Affected region(s)	Mean β-galactosidase activity (U)[b]		*cyc1-5000/CYC1* ratio (%)
		CYC1	*cyc1-5000*	
SUA8	NA[a]	53 (3)	1.24 (0.01)	2.3
sua8-1	D	29 (5)	6.3 (0.2)	22
SIT1	NA	15 (2)	0.32 (0.05)	2.1
sit1-8	D, G	8 (1)	0.38 (0.04)	4.8
sit1-278	D	4.04 (0.02)	0.76 (0.04)	19
sit1-4	F	3.8 (0.7)	0.32 (0.01)	8.4
sit1-5	F	5.1 (0.4)	0.35 (0.07)	6.9

[a] NA, not applicable.
[b] Standard deviations are indicated in parentheses.

cyc1-5000 allele were measured (Table 2). The ratio of the level of expression of the *cyc1-5000* allele to that of the wild-type *CYC1* gene increases in strains with *sua8* mutations. A low ratio, such as that measured in strains carrying a wild-type *SIT1* gene (*SUA8* or *SIT1* in Table 2), indicates that most of the transcripts that are initiated at the *cyc1-5000* promoter start upstream of the nonfunctional ATG. In contrast, a high ratio, such as that measured in a *sua8-1* mutant strain, indicates that a substantial proportion of *cyc1-5000* transcripts start downstream of the nonfunctional ATG and hence that the mutation affects the position of the transcriptional start site.

Four observations were made. First, the level of expression of the wild-type *CYC1* gene is reduced approximately two- to fourfold in *sit1* mutant strains (*sit1-8, -278, -4,* and *-5*) compared to that of a wild-type *SIT1* strain. This decrease in *CYC1* expression is not surprising, since *sit1* mutations were shown previously to affect the expression of many genes, including those, like *CYC1*, that are required for growth on nonfermentable carbon sources (8). Second, all four *sit1* mutant strains confer an increase in the ratio of *cyc1-5000* to *CYC1* expression, albeit of variable magnitude: *sit1-8* showed the smallest increase, whereas the increase conferred by *sit1-278* was comparable to that imposed by the *sua8-1* allele. Third, both region D and F mutations affect the expression ratio. Fourth, expression of the wild-type *CYC1* gene in a wild-type *SIT1* strain can be affected by the genetic background of the strain (compare *SUA8* and *SIT1* in Table 2). Whether this background difference is due to the absence of BAS1, BAS2, and GCN4 in one strain (*SIT1*) but not the other (*SUA8*) remains to be investigated. Although the *sit1* alleles tested here have a feature of *sua8* mutants (i.e., an alteration of start site selection that favors downstream start sites), we do not conclude that all of these alleles have a bona fide Sua phenotype. An Sua phenotype would require an absolute increase in the expression of *cyc1-5000*, such that this allele would be capable of supporting the growth of the strain when lactate is the carbon source (38). Our data speak only to the feature of altered start site selection that is shared by *sua8* and these *sit1* alleles.

Synthetic lethality of *sit1-8D* and *ppr2*. The clustering of *sit1* mutations in regions of RNAPII which form part of its catalytic center suggest that these mutations may affect the elongation properties of the enzyme (see Discussion). We reasoned that if the *sit1* mutations affect transcriptional elongation in vivo, this defect may be exacerbated by a deletion of the nonessential gene *PPR2*, which encodes transcription elongation factor IIS (TFIIS). This was tested by shuffling plasmids carrying the *sit1* alleles (*sit1-4, -5, -7, -9, -290, -252, -278, -8D, -8G,* and *-8DG*) into a strain carrying a disruption of both *SIT1* and *PPR2*
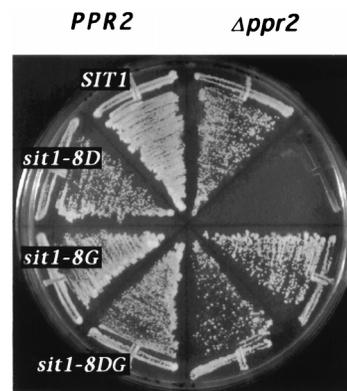

FIG. 4. Synthetic lethality of *sit1-8D* and *ppr2. TRP1 CEN/ARS* plasmids bearing *SIT1 (RPO21)* alleles (indicated by white letters on a black background) were introduced into YF2277 (*MATα rpo21::ADE2* [pJS121, *RPO21* on *URA3, CEN/ARS*]), shown on the left side of the plate or YF2278 (*MATα rpo21::ADE2 ppr2::hisG* [pJS121, *RPO21* on *URA3, CEN/ARS*]), shown on the right side of the plate, and grown on SD medium (45) lacking tryptophan and containing 5′-fluoroorotic acid for 3 days at 30°C in order to select for loss of the *URA3* maintenance plasmid (pJS121).

(YF2278) or, as a control, into a strain carrying only a disruption of *SIT1* (YF2277). In these experiments, the ability of the various *sit1* alleles to support growth in the absence or presence of TFIIS was tested by determining the ability of cells to grow on medium containing 5′-fluoroorotic acid, which selects for loss of the wild-type *SIT1* maintenance plasmid. Two observations were made. First, in the genetic background used in these experiments, the *sit1-8D* mutation is not lethal (Fig. 4). Second, this mutation is, however, lethal when combined with a deletion of *PPR2* (Fig. 4). To our knowledge, *sit1-8D* is the first mutation in a gene encoding a subunit of RNAPII, or for that matter in any gene, reported to be synthetically lethal with a *PPR2* deletion. This synthetic lethality, between a *sit1* mutation and a known elongation factor, lends further support to the notion that *sit1* mutations affect transcriptional elongation in vivo. We also noted that this synthetic lethal phenotype could be suppressed by the *sit1-8G* substitution (Fig. 4), indicating that *sit1-8G* and *PPR2* may perform a similar function. In this context, the *sit1-8G* substitution lies in a portion of the largest subunit which is close to, and may even be part of, a binding site for TFIIS (5, 50). None of the other *sit1* mutations tested (*sit1-4, -5, -7, -9, -290, -252, -278, -8G,* and *-8DG*) were synthetically lethal in combination with a *PPR2* deletion (Fig. 4, data not shown), perhaps because their effect on elongation is not as pronounced as that imposed by *sit1-8D*. The notion that the *sit1-8D* mutation imposes a more pronounced effect on transcription than the other *sit1* alleles is supported by the observation that it is the only *sit1* mutation which can be lethal.

DISCUSSION

The results presented above, which indicate that both conserved regions D and F of the largest subunit are altered by the *sit1* mutations, suggest that these mutations exert their effect by altering the overall structure of the RNAPII active center. Previous functional studies have suggested that regions D and F, together with other conserved regions of the largest and second-largest subunits of multimeric RNA polymerases, form the active site of the enzyme as broadly defined (23) to include the catalytic center together with the DNA and RNA binding sites (reviewed in references 6, 41, and 53). Region D contains a segment, NA<u>D</u>F<u>D</u>G<u>D</u>, that is invariant among multimeric

RNA polymerases (33) and is involved in binding Mg^{2+} ions, which are essential for RNA catalysis (the three aspartate residues that chelate Mg^{2+} are underlined) (54). Within the active site, region D is located very close to the transcription initiation site (54) and the 3′ end of the RNA transcript (16, 31). Cross-linking studies with *E. coli* RNAP indicated that region G is also in close proximity to the 3′ end of the transcript; while region G was the predominant cross-linking site within arrested complexes, region D was favored when cross-linking was performed on elongating complexes (31). Our finding that a region D mutation (*sit1-8D*) can be suppressed by a region G mutation (*sit1-8G*) is consistent with the notion that both regions participate in similar functions. Indeed, mutational studies have indicated that both regions are involved in the selection of the start site (13, 25; this study) and play a role in transcription elongation and termination (23, 49, 54). In *S. cerevisiae*, a mutant RNAPIII (C160-112) harboring a conditional lethal double substitution in region D (T to I at position 506 [T506I] and N509Y [Fig. 1]) was characterized biochemically (23). At a natural promoter, the mutant enzyme was as proficient as the wild-type enzyme in carrying the initiation reaction but was more prone to catalyze a slippage reaction during synthesis of the first few nucleotides. The mutant enzyme also had a markedly reduced elongation rate, which was attributable to increased pausing at intrinsic pausing sites. It was hypothesized that the double amino acid substitution could either affect the correct positioning of the 3′ end of the RNA transcript in the catalytic center of the enzyme or have a more direct effect on the formation of phosphodiester bonds (23). One of the two substitutions (N509Y) affects a residue of the invariant Mg^{2+}-binding motif and was found to be lethal unless suppressed by the second region D substitution (T506I) (23). This is reminiscent of the *sit1-8D* substitution (N479Y), which changes the analogous residue in the invariant motif of RNAPII (Fig. 1) and can also be lethal unless suppressed by the *sit1-8G* substitution (A1076V).

Region F, the other region altered by *sit1* mutations, forms a part of the enzyme active site which is targeted by certain inhibitors. Region F can be mutated to confer resistance to α-amanitin (reviewed in reference 6), which inhibits the translocation step of eukaryotic RNAPII (21), and to the antibiotic streptolydigin (44, 51), which inhibits the elongation phase of prokaryotic RNAP (18, 32, 48). A role for region F in transcriptional elongation and translocation is also supported by studies of mutant RNAPs. One *Drosophila melanogaster* RNAPII mutant (C4) bearing an α-amanitin resistance substitution in region F (R741H) (Fig. 1) was found to elongate more slowly in vitro and have an increased apparent K_m for UTP (20). In yeast, a mutant RNAPIII (C160-270) that carried a double substitution in region F (D829A R830A) (Fig. 1) and was defective in the transition from abortive initiation to elongation and in its ability to exit from pause sites was isolated (47). The mutant enzyme also showed an increased RNA cleavage activity in halted ternary complexes. Finally, in *E. coli*, several region F mutations were isolated that increase termination in the *trp* operon leader region or decrease termination at a ρ-independent terminator in vivo (49). These mutations also affected termination efficiency at other ρ-independent terminators in vitro, although the magnitude and direction of the effect appeared to be dependent on the type of terminator used (49). The last of these observations raise the possibility that the effect of *sit1* mutations on transcription varies from one gene to another. Indeed *sit1* mutations can lead to both over- and underexpression of genes such as *HIS4* and *CYC1* (Table 2), respectively. These profound effects on gene expression most

likely underlie the many pleiotropic phenotypes of *sit1* mutants.

Further support for the notion that *sit1* mutations alter the active site of RNAP comes from experiments in which the Mg^{2+} ion of the enzyme was replaced by Fe^{2+}. Hydroxyl radicals generated by Fe^{2+} cleave protein and DNA in a 1-nm radius of the ion. In these experiments, DNA is cleaved immediately upstream of the transcriptional start site (54), indicating that the Mg^{2+} ion is near or at the catalytic center of the enzyme. In a similar fashion, cut sites in the RNAP peptide chain also identified residues that are in the active-site area (35). β′ was cleaved in three regions: conserved regions D, F, and G. These regions correspond to those in which the *sit1* mutants and a suppressor to the growth defect of a *sit1* mutation (*sit1-8G*) were identified.

Two lines of evidence suggest that the pleiotropic effects of *sit1* alleles can vary according to the genetic background of the strain in which they are expressed. First, in at least one instance, we have shown that the Sit phenotype can be semidominant, whereas in other genetic backgrounds it is recessive. Second, we found that the *sit1-8D* substitution is lethal in one genetic background, whereas in another it is viable. Although we do not yet fully understand which set of genes is involved in these background differences, we nevertheless have gathered evidence to suggest that at least one gene, *PPR2*, which encodes the transcription elongation factor TFIIS, can affect the phenotype imposed by the *sit1-8D* substitution. We found that in a genetic background in which it is normally viable, the *sit1-8D* allele can be synthetically lethal in combination with a disruption of the nonessential gene *PPR2* and that this synthetic phenotype can be suppressed by the *sit1-8G* substitution which lies in, or close to, a TFIIS-binding site in RNAPII (5, 50). These findings have two implications. First, they raise the possibility that some of the differences we observed between genetic backgrounds are due to differences in genes which encode proteins that can affect transcriptional elongation, either directly or indirectly. Further analysis of these genetic differences should reveal whether this hypothesis is correct. Second, and most importantly, the genetic connection between *sit1-8D*, *sit1-8G*, and a known transcription elongation factor lends further support to the hypothesis that *sit1-8D* affects the elongation properties of RNAPII in vivo.

Other than mutations that truncate the C-terminal domain of the largest subunit of RNAPII (2, 42), the *sit1* mutations are the only mutations that implicate RNAPII directly in the process of transcriptional activation. Our finding that *sit1* substitutions affect start site selection and occur in regions of RNAPII that form the enzyme active center implies that there is an intimate relationship between initiation and elongation and has implications for the mechanism of transcriptional activation. To our knowledge, our findings provide the first evidence in eukaryotes that alteration of the enzyme catalytic center can affect transcriptional activation. Previous studies of the mechanism of transcriptional activation have suggested that activation domains stimulate transcription by several mechanisms, including counteracting the repressing effect of chromatin, recruiting the RNAPII holoenzyme complex to the promoter through multiple contacts between activation domains and components of the RNAPII holoenzyme, and/or increasing the processivity of elongating RNAPII (reviewed in references 12 and 46). The results presented in this study provide genetic evidence that partial activation of transcription can also occur by alteration of the RNAPII active site and suggest that some transcription-regulatory proteins exert their effect by modifying, either directly or indirectly, the RNAPII active center. In *E. coli*, the conformation of the RNAP active center and in

particular the way in which it contacts the nascent RNA and the DNA template are major determinants of the processivity of the enzyme (reviewed in reference 29), which can be regulated by antitermination factors such as the N and Q gene products of phage lambda (reviewed in reference 24). By analogy, eukaryotic regulatory proteins that are known to increase the processivity of RNAPII, such as human immunodeficiency virus type 1 Tat, yeast GAL4, human p53 and E2F1, and herpes simplex virus VP16 (14, 28, 52), could exert their effect by altering, either directly or indirectly, the structure or conformation of the enzyme active site during elongation. *sit1* mutations, by directly affecting the RNAPII active center, could help stabilize a more processive form of the enzyme and in doing so partially bypass the need for promoter-bound transcriptional activators at *HIS4*.

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