MOLECULAR AND CELLULAR BIOLOGY, Nov. 1991, p. 5781–5791
0270-7306/91/115781-11$02.00/0

Vol. 11, No. 11

Mutations in a Conserved Region of RNA Polymerase II Influence the Accuracy of mRNA Start Site Selection

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Received 11 June 1991/Accepted 29 August 1991

A sensitive phenotypic assay has been used to identify mutations affecting transcription initiation in the genes encoding the two large subunits of *Saccharomyces cerevisiae* RNA polymerase II (*RPB1* and *RPB2*). The *rpb1* and *rpb2* mutations alter the ratio of transcripts initiated at two adjacent start sites of a δ-insertion promoter. Of a large number of *rpb1* and *rpb2* mutations screened, only a few affect transcription initiation patterns at δ-insertion promoters, and these mutations are in close proximity to each other within both *RPB1* and *RPB2*. The two *rpb1* mutations alter amino acid residues within homology block G, a region conserved in the large subunits of all RNA polymerases. The three strong *rpb2* mutations alter adjacent amino acids. At a wild-type promoter, the *rpb1* mutations affect the accuracy of mRNA start site selection by producing a small but detectable increase in the 5′-end heterogeneity of transcripts. These RNA polymerase II mutations implicate specific portions of the enzyme in aspects of transcription initiation.

Accurate initiation of mRNA synthesis is essential for appropriate gene expression. However, the basic process by which RNA polymerase II initiates synthesis of an mRNA transcript from a defined start site is only poorly understood. In *Escherichia coli*, the site of transcription initiation is rigidly restricted, primarily through the interaction of the σ subunit of RNA polymerase with conserved promoter sequences and secondarily through the purine nucleotide triphosphate preference of the active site of the enzyme (28, 29, 52, 60, 82). In eucaryotes, general transcription factors that assemble at TATA elements are important for positioning RNA polymerase II near the approximate transcriptional start site (for reviews, see references 54 and 69), but the roles that RNA polymerase II and additional factors play in determining the precise mRNA start site are unclear.

Since purified RNA polymerase II does not initiate transcription accurately in vitro (71), the additional *cis*- and *trans*-acting factors that contribute to accurate transcription initiation have been the subject of considerable investigation. Transcription is initiated approximately 30 bp downstream from a TATA element in higher eucaryotic promoters (11) and 60 to 120 bp in *Saccharomyces cerevisiae* promoters (32). Mutagenesis of the TATA box can produce wide spacing (macroheterogeneity) of mRNA 5′ termini (19, 30, 47, 50), and changing the position of the TATA element can result in a corresponding shift in the position of the mRNA start site (31, 47, 53). Consequently, the TATA box is believed to serve as a guidepost from which the transcriptional apparatus can approximately measure the mRNA start site. A single polypeptide, TFIID, binds specifically to the TATA box, promoting the subsequent assembly of the other general transcription factors and RNA polymerase II that form the preinitiation complex (12, 51).

Eucaryotic mRNAs are often initiated at multiple sites within a small region of the template (5, 32, 43). This microheterogeneity, which is particularly common in *S. cerevisiae*, may be due to the ability of the promoter-associated RNA polymerase II to scan a small region of the promoter for a thermodynamically favorable start site. Sequences surrounding the mRNA start site are known to influence the position of transcription initiation at eucaryotic promoters, particularly in *S. cerevisiae* (16, 34, 47, 53) and at the TATA-less promoters of higher eucaryotes (63, 64). It is not known whether RNA polymerase II interacts with the initiation region on its own or in concert with some additional factor and whether the interaction is sequence specific.

S. cerevisiae is an attractive organism in which to study the role of RNA polymerase II in mRNA start site selection. The genes for all *S. cerevisiae* RNA polymerase II subunits have been cloned, facilitating the molecular genetic analysis of these polymerase components (2, 37, 40, 66, 75–81). The subunit architecture of RNA polymerase II is relatively similar among eucaryotes (54, 58, 80). This conservation is also reflected at the molecular level: sequence analysis has revealed that the two largest subunits of all eucaryotic RNA polymerase II enzymes studied thus far contain multiple segments of striking homology to regions in β′ and β, the two large subunits of *E. coli* RNA polymerase (for a review, see reference 20). Furthermore, a number of general (13, 14, 33) and gene-specific (39, 42, 70) *S. cerevisiae* transcription factors have been shown to function with mammalian polymerase. The conservation of these various components of the transcriptional machinery indicates that the process of RNA polymerase II transcription initiation is fundamentally similar in all eucaryotes.

In this study, we have used a sensitive phenotypic assay to identify mutations in the *S. cerevisiae* RNA polymerase II large-subunit genes, *RPB1* and *RPB2*, which alter transcription initiation patterns at specific promoters. The *rpb1* mutations occur in homology block G, a region that is highly conserved in RNA polymerases. Cross-linking studies with *E. coli* RNA polymerase suggest that homology block G is located near the enzyme's active site (10). The results described here indicate that this segment of *S. cerevisiae* RNA polymerase II has an influence on the accuracy of mRNA start site selection.

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TABLE 1. Yeast strains

Strain	Genotype
Z92	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pRP112)
Z93	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pRP114)
Z94	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pDH14)
Z192	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pDH15)
Z193	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pRP112, pRP114)
Z194	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pRP112, pDH14)
Z195	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pRP112, pDH15)
Z196	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112)
Z197	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP114)
Z198	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pDH14)
Z199	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pDH15)
Z200	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112, pRP114)
Z201	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112, pDH14)
Z202	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112, pDH15)
Z26	*MATα ura3-52 leu2-3,112 his3Δ200 rpb1Δ187::HIS3*(pRP112)
Z27	*MATα ura3-52 leu2-3,112 his3Δ200 rpb1Δ187::HIS3*(pRP114)
8930-2C	*MATa ura3-52 his4-912δ spt3-2*
Z203	*MATα ura3-52 leu2-3,112 his3Δ200 his-912δ lys2-128δ rpb1Δ196::HIS3*(pDH26)
Z204	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ196::HIS3*(pDH27)
Z205	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ*
Z206	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1-501*
Z207	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1-502*
Z208	*MATα ura3-52 leu2-3,112 his4-912δ lys2-128δ*
Z209	*MATα ura3-52 leu2-3,112 his4-912δ lys2-128δ rpb1-501*
Z210	*MATα ura3-52 leu2-3,112 his4-912δ lys2-128δ rpb1-502*
Z211	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912 lys2-128δ rpb1Δ196::HIS3*(pRP112)
Z212	*MATα ura3-52 leu2-3,112 his3Δ200 his4-917δ lys2-128δ rpb1Δ196::HIS3*(pRP112)
Z214	*MATa ura3-52 leu2-3,112 his3Δ200 his4-917 rpb1Δ187::HIS3*(pRP112)
Z215	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-173r2 rpb1Δ196::HIS3*(pRP112)
Z224	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb2Δ297::HIS3*(pRP212)
Z225	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb2Δ297::HIS3*(pRP214)
Z226	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb2Δ297::HIS3*(pDH17)
Z227	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb2Δ297::HIS3*(pDH18)
Z228	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb2Δ297::HIS3*(pDH19)
Z229	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb2Δ297::HIS3*(pDH20)
Z230	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb2Δ297::HIS3*(pDH21)
Z231	*MATa ura3-52 leu2-3,112 his3Δ200 his4-912 lys2-128δ rpb2Δ297::HIS3*(pRP212)
Z232	*MATα ura3-52 leu2-3,112 his3Δ200 his4-917δ lys2-128δ rpb2Δ297::HIS3*(pRP212)
Z233	*MATa ura3-52 leu2-3,112 his3Δ200 his4-917 lys2-128δ rpb2Δ297::HIS3*(pRP212)
Z234	*MATa ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-173r2 can1 rpb2Δ297::HIS3*(pRP212)
Z450	*MATα ura3-52 leu2-3,112 his3Δ200 rpb1Δ187::HIS3*(pRP114)
Z451	*MATα ura3-52 leu2-3,112 his3Δ200 rpb1Δ187::HIS3*(pDH14)
Z452	*MATα ura3-52 leu2-3,112 his3Δ200 rpb1Δ187::HIS3*(pDH15)
Z456	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112, pDH60)
Z457	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112, pDH64)
Z458	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112, pDH63)
Z459	*MATα ura3-52 leu2-3,112 his3Δ200 his4-912δ lys2-128δ rpb1Δ187::HIS3*(pRP112, pDH67)
N420	*MATa ura3-52 leu2-3,112 his3Δ200 rpb1Δ187::HIS3*(pC3)

MATERIALS AND METHODS

Yeast strain constructions. Yeast strains used in this study are listed in Table 1. *rpb1Δ196::HIS3* and *rpb1Δ187::HIS3* have been described previously (49). Strains Z93, Z94, and Z192 to Z195 are isogenic derivatives of Z92 produced by plasmid shuffle mutagenesis. Strains Z197 to Z202 were similarly derived from Z196. Plasmids pDH14 and pDH15 are hydroxylamine-mutagenized derivatives of pRP114 which carry the *rpb1-501* and *rpb1-502* alleles, respectively. Strains Z225 to Z230 were derived from Z224 through plasmid shuffle mutagenesis. Plasmids pDH17 through pDH21 carry the alleles *rpb2-501* through *rpb2-505* respectively. Z205, Z206, and Z207 are 5-fluoroorotic acid (5-FOA) resistant, *his3* derivatives of Z92, Z203, and Z204 which have integrated the corresponding plasmid-derived *RPB1* alleles into the chromosomal locus. Z208, Z209, and Z210 are *HIS3* derivatives of Z205, Z206 and Z207, respectively, produced by transformation of the corresponding strains with *Eag*I-linearized pDH40, followed by selection with 5-FOA.

Strains Z450, Z451, and Z452 were derived from Z26 by plasmid shuffle. Z456 to Z459 are transformants of Z196. Strains Z27 (49), N420 (49), and 8930-2C (72) were provided by M. Nonet and G. R. Fink.

Yeast transformations were performed by the lithium acetate method of Ito et al. (38). Integrative transformation was verified by Southern analysis, as described by Boeke et al. (8). Routine genetic manipulations were performed as described in Sherman et al. (59). Synthetic medium containing dextrose (SD) and synthetic complete medium (SC) were prepared as previously described (59). 5-FOA plates contained 1 g of 5-FOA per liter, as recommended by Boeke et al. (9).

Plasmid constructions. Routine DNA manipulations were performed essentially as described in Maniatis et al. (44). pRP112 and pRP114 are centromere plasmids carrying both *RPB1* and either *URA3* or *LEU2*, respectively (49). pRP116 consists of an *Aat*II-*Nar*I *RPB1* fragment from pRP19 (48) inserted into *Aat*II-*Nar*I-cut YEp24 (47a). Plasmids pRP212 and pRP214 are centromere plasmids carrying both *RPB2* and either *URA3* or *LEU2*, respectively (57). Plasmid pDH40 contains a *Bam*HI fragment of *HIS3* inserted into YIp5 such that *HIS3* and *URA3* are transcribed divergently.

Plasmids pDH14, pDH15, and pDH17 to pDH21 were isolated from strains Z94, Z192, and Z226 to Z230, respectively, by the procedure of Hoffman and Winston (36). A 2.9-kb *Xba*I-*Hin*dIII *rpb1-501* fragment was isolated from pDH14 and inserted into M13mp18 to create ϕDH59. ϕDH62, an *rpb1-501,502* derivative of ϕDH59, was made by using the oligonucleotide 5′ CACAAATGATCCTTAAC ACC 3′ and the Amersham oligonucleotide-directed in vitro mutagenesis system (version 2) according to the manufacturer's directions. Sequence analysis was performed with a Sequenase kit (United States Biochemical Corporation), using a 20-mer oligonucleotide comprising the sequence from +3453 to +3476 (2) of *RPB1*. pDH60 was prepared by cutting pRP114 with *Nco*I and *Pvu*II, filling in the 5′ overhang with Klenow fragment, and religating. Plasmids pDH64, pDH67, and pDH63 were then constructed by inserting the *Xba*I-*Hin*dIII internal *rpb1-501*, *rpb1-502*, or *rpb1-501,502* fragments from pDH14, pDH15, or ϕDH62, respectively, into pDH60. Two independent pDH63 isolates, constructed in this manner, gave identical results in the assay described in Fig. 7.

Riboprobe constructs for Northern blot (RNA blot) analysis were prepared as follows. A 1.2-kb *Sal*I-*Bgl*II internal fragment of *HIS4* was inserted into *Sal*I-*Bam*HI-cut pUC18 to create pDH24. pBC2 was constructed by placing a *Kpn*I-*Pst*I *HIS4*-carrying fragment from pDH24 into *Kpn*I-*Pst*I-cut pGEM3 (Promega). pDH35 was prepared by inserting a 1.65-kb *Bam*HI-*Hin*dIII *ACT1* fragment into *Bam*HI-*Hin*dIII-cut pGEM4.

A 1.45-kb *Sal*I-*Pvu*II fragment which carried the *his4-912*δ promoter and early coding region was isolated from YCp701 (35) and cloned into *Sal*I-*Sma*I-cut pUC18 to produce plasmid pDH23. This same region was removed from pDH23 as an *Eco*RI-*Sal*I fragment and inserted into *Eco*RI-*Sal*I-cut M13mp19 replicative-form DNA to create ϕDH44. A 1.1-kb *Sal*I-*Pvu*II fragment carrying the *HIS4* promoter and early coding region was cloned into *Sal*I-*Sma*I-cut pUC18 to produce plasmid pDH22. A 370-bp *Xho*I-*Sph*I fragment carrying a region of the *HIS4* early coding region was then isolated from pDH22 and inserted into *Sal*I-*Sph*I-cut pGEM3 to create pDH45.

Plasmid shuffle mutagenesis. Hydroxylamine-mutagenized pRP114 and pRP214 DNA, prepared essentially as described by Nonet et al. (48), was obtained from C. Scafe. The *RPB1* and *RPB2* knockout frequencies of the amplified libraries were estimated to be 2 and 1%, respectively.

Recessive mutations in *RPB1* which lead to suppression of *his4-912*δ and *lys2-128*δ were isolated by a variation on the basic scheme of plasmid shuffling (9). Strain Z92 was transformed with an amplified library of hydroxylamine-mutagenized DNA from pRP114. Merodiploid transformants, which carried both pRP112 and mutagenized pRP114, were replica plated to 5-FOA plates to select for loss of pRP112. Both the merodiploid and haploid transformants were tested by replica plating for growth in the absence of either histidine or lysine. His^+ and Lys^+ phenotypes exhibited by transformants lacking pRP112 resulted from mutations in the *RPB1* gene on the mutagenized pRP114 plasmid. All incubations were performed at room temperature (approximately 23°C). Plasmid DNA was isolated from strains thought to harbor an *spt* mutation recessive to pRP112 by the rapid procedure of Hoffman and Winston (36) and subsequently passaged through *E. coli*. Linkage of the recessive *spt* mutation to the *RPB1* plasmid DNA was determined by the same plasmid shuffle scheme used initially to detect the mutations. Plasmids pDH14 and pDH15, which were isolated from strains Z94 and Z192, respectively, were subsequently shuffled, in an analogous manner, into strain Z196 to produce strains Z198 and Z199. Suppression of *his4-912*δ and *lys2-128*δ was stronger in this background than that of Z92. Similar differences have been observed previously between strains carrying the complete (*Δ187*) and partial (*Δ196*) deletions of the *RPB1* gene (49). In an analogous screen for *spt* mutations in *RPB2*, strain Z224 was transformed with hydroxylamine-mutagenized, amplified pRP214 DNA.

RNA isolation and Northern analysis. Yeast strains were grown at 22°C to mid-log phase (optical density at 600 nm [OD_{600}] = 0.5) in 10 ml of either SD containing Ura, His, and Lys or SD containing His and Lys (merodiploids) unless otherwise indicated. For derepression of GCN4, 3-aminotriazole was added to a final concentration of 10 mM when the cultures had attained an OD_{600} of approximately 0.1, and the cultures were harvested 15 h later. Total cellular RNA was prepared from these cultures as described by Scafe et al. (57).

Portions (5 μg each) of the total-RNA samples were subjected to denaturing agarose gel electrophoresis in the presence of formaldehyde essentially as described by Maniatis et al. (44). Gels were 1.2% agarose. Northern hybridization and wash conditions were similar to those described by Meinkoth and Wahl (46). Hybridization was done overnight at 55°C in a solution containing 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 8.0]), 0.1% sodium dodecyl sulfate (SDS), and 250 μg of denatured salmon sperm DNA per ml. ^{32}P-labelled *HIS4* and *ACT1* riboprobes were prepared from linearized pBC2 and pDH35, respectively, using a Riboprobe Gemini System II kit (Promega). The ^{32}P-labelled *ACT1* riboprobe was diluted 10-fold with unlabelled *ACT1* riboprobe. The filters were washed at high stringency (0.1× SSPE–0.1% SDS, 65°C).

S1 nuclease analysis. S1 nuclease analysis of *HIS4* transcripts was performed, with some modifications, as described by Favalaro et al. (27). Twenty-five micrograms of each total yeast RNA sample and 0.25 μg of ϕDH44 were combined in 6 μl of 5× hybridization buffer [2 M NaCl, 0.2 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.5), 1 mM EDTA], and 24 μl of deionized formamide was added to the buffer. Samples were denatured by heating for 15 min at 80°C and were then transferred immediately to

41°C for overnight hybridization. Three microliters of S1 buffer (300 mM sodium acetate [pH 4.5], 280 mM NaCl, 4.5 mM zinc acetate) at 4°C containing 3 μg of denatured salmon sperm DNA and 1,000 U (1985 designation) of Boehringer Mannheim S1 nuclease was added to each sample, and each sample was transferred to 37°C for 30 min. Protected DNA-RNA hybrids were recovered by ethanol precipitation and subjected to 2% agarose–Tris-borate-EDTA (TBE) gel electrophoresis and Southern analysis. A ^{32}P-labelled riboprobe homologous to the 3′ 370 bp of ϕDH44, prepared from pDH45, was used as the hybridization probe. Washes were done at 55°C in 0.1× SSPE–0.1% SDS.

Primer extension analysis. The *HIS4* primer was prepared by polynucleotide kinase (New England BioLabs) labelling of an oligonucleotide complementary to sequences +1426 to +1456 of *HIS4* (21). Twenty micrograms of total RNA was hybridized with the labelled primer and extended with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) essentially as described by Lillie et al. (41). Hybridization and extension reactions were done at 37°C. The reaction products were resolved by 6% polyacrylamide–urea sequencing gel electrophoresis.

Mapping *rpb1* and *rpb2* mutations. Mutations *rpb1-501* and *rpb1-502* were mapped functionally by a gap repair procedure. Gapped centromere- or 2μm-based *URA3 RPB1* plasmids lacking various portions of the *RPB1* gene were produced by restriction endonuclease digestion of plasmid pRP112 or pRP116. pRP112 was digested either with *Aat*II and *Xba*I or with *Xba*I and *Sna*BI. Because pRP112 contains a *Bgl*II site within its centromere region (45), pRP116, rather than pRP112, was digested either with *Bgl*II or with *Bgl*II and *Xba*I. Competent Z197, Z198, and Z199 were transformed directly with melted SeaPlaque agarose (FMC BioProducts)–TAE (Tris-acetate-EDTA) gel slices containing the gapped plasmids. For each gapped plasmid/Z198 or Z199 transformation, 100 transformants were tested for growth on SC lacking both Ura and Leu, SC lacking Ura, Leu, and His, and SC lacking Ura, Leu, and Lys.

Mutations *rpb2-501* to *rpb2-505* were mapped as follows. The 3-kb *Xba*I-*Xho*I or 3.5-kb *Eco*RI-*Sal*I fragments from plasmids pDH17 to pDH21 were subcloned into pRP214. Strain Z224 was then transformed with the chimeric plasmids and tested for growth on SC lacking Leu and His and on SC lacking Leu and Lys following loss of pRP212 as described above.

The various plasmids were then subjected to DNA sequence analy is in the regions where the mutations were shown to reside by the previous mapping experiments: between the *Xba*I and proximal *Bgl*II sites for pDH14 and pDH15, between the *Xba*I and *Eco*RI sites for pDH17, and between the *Eco*RI and *Xho*I sites for plasmids pDH18 to pDH21. Two-microgram samples of plasmid DNAs were prepared for sequence analysis essentially as described by Chen and Seeburg (15). 20-mer oligonucleotides spanning the various regions were used as primers. DNA sequencing was performed by using a Sequenase kit (United States Biochemical Corporation). Denaturing acrylamide gel electrophoresis was done essentially as described by Biggin et al. (6). For *rpb2-503*, two missense mutations were discovered in the region sequenced. The G-A change at position 1790 (66) was determined to be the functional one, because a gap-repaired pRP212 derivative carrying only this mutation lead to suppression of both *his4-912*δ and *lys2-128*δ.

Western blot (immunoblot) analysis. Strain N420 (49) produces a truncated, but nonetheless functional, RPB1. N420 was transformed with plasmids pDH60, pDH64, pDH67, and pDH63. The resultant strains were grown in SC lacking both uracil and leucine and harvested at mid-log phase (OD_{600} = 0.5). The cell pellets were resuspended in the extraction buffer of Valenzuela et al. (68), and crude protein extracts were prepared by vortexing in the presence of glass beads. Samples (20 and 10 μg) of total protein from each sample were subjected to electrophoresis on a 7.5% SDS–polyacrylamide gel with a low (0.07%) *N′,N′*-bisacrylamide cross-linker concentration as described by Dreyfuss et al. (22). Electrotransfer of protein to nitrocellulose was performed as described by Towbin et al. (67), using transfer buffer (20 mM Tris [pH 8.0], 144 mM glycine, 25% methanol, 0.01% SDS). The ProtoBlot immunoscreening system (Promega) was used according to the manufacturer's instructions except that antibody incubations were done with 1× ANT (50 mM Tris [pH 8.0], 150 mM NaCl, 0.02% NaN_3)–5% nonfat dry milk, and washes were done with 1× ANT–0.05% Tween 20. The anti-RPB1 carboxy-terminal repeat monoclonal antibody 8WG16, obtained from N. Thompson and R. Burgess, was diluted 1:1,000.

RESULTS

Mutations in both *RPB1* and *RPB2* can suppress δ-insertion mutations. Our screen for RNA polymerase II mutations affecting transcription initiation is based on the expectation that such mutations could restore the expression of two genes, *HIS4* and *LYS2*, each rendered inactive by a δ-element insertion. These insertions were produced by the transposition of a Ty retrotransposon into the *HIS4* pro-

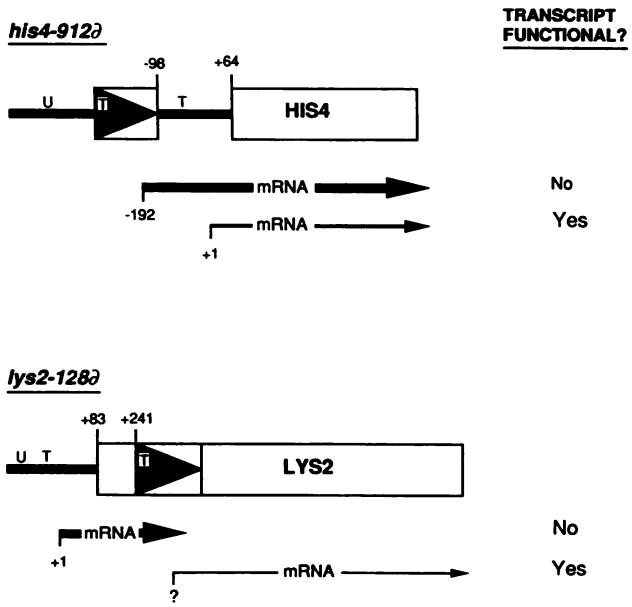

FIG. 1. *his4-912*δ and *lys2-128*δ and their transcripts. The approximately 330-bp δ-element insertions in *HIS4* and *LYS2* are indicated by boxed arrows. *HIS4* and *LYS2* protein-coding segments are shown as open boxes. In both δ-insertion promoters, +1 corresponds to the position at which wild-type (*HIS4* or *LYS2*) transcripts are initiated. The δ-initiated *his4-912*δ transcript is not translated into functional HIS4, probably as a consequence of in-frame start and stop codons preceding the HIS4 translational start (18). The *lys2-128*δ transcript initiated at +1 terminates prematurely (probably at the δ-element termination site) and, consequently, is nonfunctional. The 5′ end of the downstream *lys2-128*δ transcript has not been mapped precisely. U, UAS; T, TATA box.

moter or *LYS2* early coding region, followed by homologous recombination between the δ-element terminal repeat sequences of the Ty element (7). The structure of the altered promoter region is essentially the same for both insertion mutations (*his4-912δ* and *lys2-128δ*): a UAS region followed by two potential mRNA start sites (Fig. 1). Strains carrying these insertions are histidine and lysine auxotrophs, because *HIS4* and *LYS2* transcripts are initiated predominantly at the first of the two start sites, producing nonfunctional messages (18, 35, 61). It has been observed previously that mutations in a number of unlinked *SPT* (suppressor of Ty) genes can revert the His^- and Lys^- phenotype of such a δ-insertion strain by increasing the levels of transcripts initiated at the wild-type *HIS4* start site and an undefined downstream *lys2-128δ* site, respectively (17, 18, 23, 65, 74).

We have obtained *spt* mutations in *RPB1* and *RPB2* which suppress the *his4-912δ* and *lys2-128δ* mutations. A plasmid shuffle screen (9) of 15,000 colonies, each carrying a mutagenized *RPB1* plasmid, yielded two *spt* mutations, *rpb1-501* and *rpb1-502*, which allow the δ-insertion strain to grow in the absence of histidine and lysine (Fig. 2). *RPB1/rpb1-501* and *RPB1/rpb1-502* merodiploids are His^- and Lys^-, indicating that both *rpb1* mutations are recessive. Haploid strains containing *rpb1-501* or *rpb1-502* integrated into the chromosomal *RPB1* locus (see Materials and Methods) are His^+ and Lys^+ (data not shown).

An analogous plasmid shuffle screen of 7,000 transformants carrying mutagenized *RPB2* plasmid DNA yielded five plasmid-linked mutations that suppress *his4-912δ* or *lys2-128δ*. Unlike their *RPB1* counterparts, the *RPB2* mutations *rpb2-501* to *rpb2-505* do not suppress *his4-912δ* and *lys2-128δ* to the same extent (Table 2). *rpb2-503*, *rpb2-504*, and *rpb2-505* strongly suppress both insertions. On the other hand, *rpb2-501* suppresses *his4-912δ*, but not *lys2-128δ*, whereas *rpb2-502* strongly suppresses *lys2-128δ* but poorly suppresses *his4-912δ*. Phenotypically, *rpb2-503* to *rpb2-505* are strong suppressors, while *rpb2-501* and *rpb2-502* are relatively weak. In addition, *RPB2/rpb2-503*, *RPB2/rpb2-504*, and *RPB2/rpb2-505* merodiploids suppress to some degree the His^- phenotype conferred by *his4-912δ* (data not shown).

TABLE 2. Allele specificities of *rpb1* and *rpb2* mutations

Mutation	Ability to express the indicated Ty or δ insertion					
	HIS4				*LYS2*	
	δ912	*Ty912*	*δ917*	*Ty917*	*α128*	*Ty173r2*[a]
rpb mutations						
rpb1-501	+	−	−	+	+	−
rpb1-502	+	−	−	+	+	−
rpb2-501	+	−	−	−	−	−
rpb2-502	+/−	−	−	−	+	−
rpb2-503	+	−	−	−	+	−
rpb2-504	+	−	−	+/−	+	−
rpb2-505	+	−	−	+/−	+	−
spt mutations[b]						
Class 1 (*spt3*, *spt7*, *spt8*, *spt15*)	+	−	+	+	+	+
Class 2 (*spt2*, *spt4*, *spt5*, *spt6*, *spt11*, *spt12*)	+	−	−	+/−[c]	+	−
Class 3 (*spt13*, *spt14*)	−	−	−	+	−	−

[a] An unsuppressed *lys2-173r2* strain is Lys^+; when suppressed, it is Lys^-.
[b] Allele-specificity patterns of class 1, 2, and 3 *spt* mutations were compiled from the results in references 23, 26, 62, and 72 to 74.
[c] The degree of suppression of *his4-917* by class 2 *spt* mutations is both gene and allele specific (71a, 72).

Each of the *rpb1* and *rpb2* mutations was assayed for its ability to suppress a series of Ty and δ insertions at *HIS4* and *LYS2*. The resulting patterns of allele specificity (Table 2) demonstrate that the *rpb1* and *rpb2* mutations are similar to those in a subset of *SPT* genes which we refer to as class 2 *spt* mutations. In contrast to several of the other class 2 *spt*

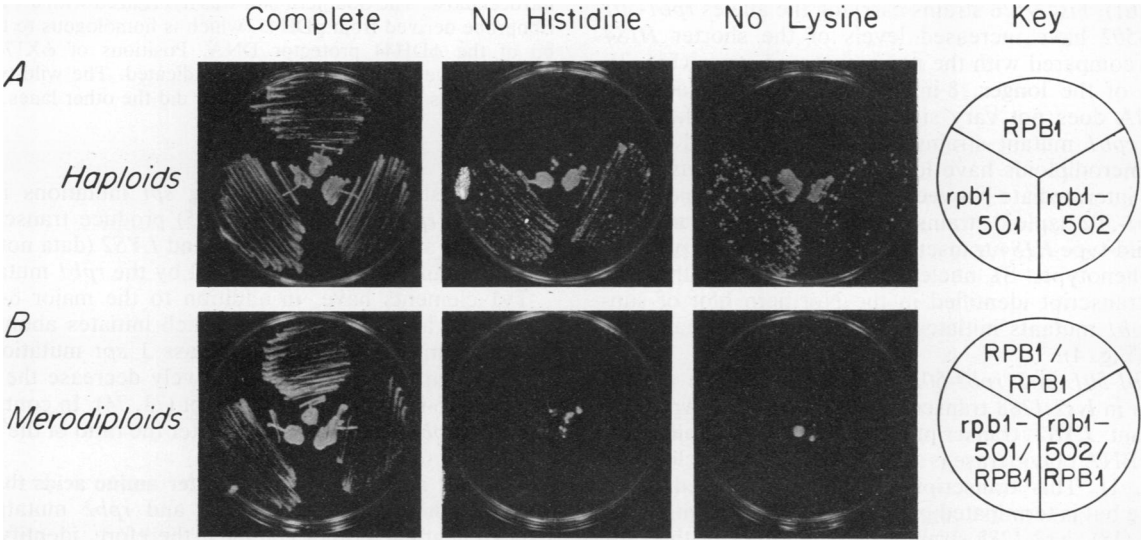

FIG. 2. Phenotypic suppression of *his4-912δ* and *lys2-128δ* by the *rpb1* mutations. Growth of isogenic *RPB1*, *rpb1-501*, and *rpb1-502* haploid strains Z93, Z94, and Z192 (A) was compared with that of the corresponding merodiploid strains Z193, Z194, and Z195 (B). Complete plates (SC lacking Leu or Ura and Leu) were incubated for 2 days, plates lacking histidine (SC lacking Leu and His or Ura, Leu, and His) were incubated for 7 days, and plates lacking lysine (SC lacking Leu and Lys or Ura, Leu, and Lys) were incubated for 9 days. All incubations were done at room temperature.

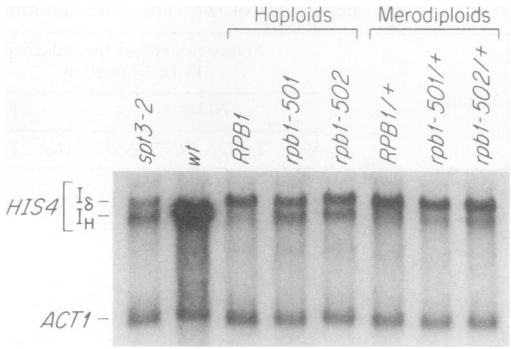

FIG. 3. Northern analysis of *his4-912δ* transcripts in isogenic wild-type (wt) and *rpb1* mutant strains. Total RNA isolated from strains 8930-2C, Z27, Z197, Z198, Z199, Z200, Z201, and Z202 (left to right) was subjected to denaturing gel electrophoresis and transferred to nitrocellulose. The Northern blot was probed with ^{32}P-labelled *HIS4* and *ACT1* riboprobes derived from pBC2 and pDH35, respectively. The two *HIS4* transcripts produced in the *spt3-2* strain, which initiate within 912δ (I_δ) and at the wild-type *HIS4* site (I_H), are included as size standards. The *ACT1* transcript serves as a normalization standard for amount of RNA.

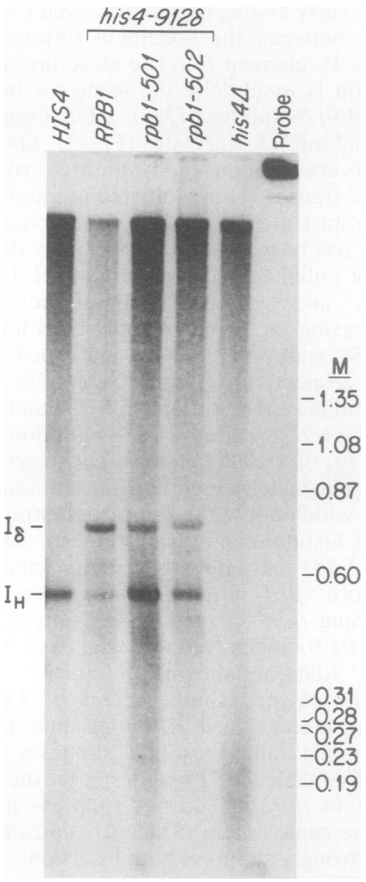

FIG. 4. S1 nuclease analysis of *his4-912δ* transcripts in isogenic wild-type and *rpb1* mutant strains. Total RNA from strains Z27, Z197, Z198, Z199, and Z223 (left to right) was hybridized to single-stranded φDH44 DNA, which is homologous to the *his4-912δ* promoter and early coding region. Hybridization mixes were then treated with S1 nuclease. Nuclease-resistant hybrids were subjected to electrophoresis on a 2% agarose–TBE gel and transferred to nitrocellulose. The Southern blot was hybridized with a ^{32}P-labelled riboprobe derived from pDH45, which is homologous to the 3′ 370 bp of the φDH44 protector DNA. Positions of φX174 *Hae*III molecular weight markers (M) are indicated. The wild-type *HIS4* lane contains 1/10th as much RNA as did the other lanes.

mutations, the *spt* mutations in *RPB1* and *RPB2* have no significant effect on growth rate at temperatures ranging from 12 to 38°C, nor do they affect mating and sporulation efficiencies (data not shown). These *rpb1* mutations do not cause an increase in the levels of *SPT6* (*SSN20*), *SPT5*, or *HTB1* (which encodes histone H2B) RNAs (data not shown); an increase in the level of any of these RNAs can lead to suppression of δ insertion mutations (17, 18, 65).

Mutant polymerases display altered transcription initiation patterns at δ-insertion promoters. Northern and S1 nuclease analysis of *his4-912δ* transcripts demonstrate that the mRNA initiation pattern is altered in the *rpb1* mutant strains. *his4-912δ* strains with wild-type RNA polymerase produce two transcripts, a major transcript initiated in the δ-element and a shorter, minor transcript initiated at the normal *HIS4* start site (61). *his4-912δ* strains carrying the alleles *rpb1-501* and *rpb1-502* have increased levels of the shorter *HIS4* transcript compared with the nonsuppressed strain (Fig. 3). The level of the longer, δ-initiated, transcript relative to *ACT1* RNA does not vary significantly between the wild-type and *rpb1* mutant strains. *RPB1/rpb1-501* and *RPB1/rpb1-502* merodiploids have levels of the shorter *his4-912δ* transcript intermediate between that of the *RPB1* and *rpb1-501* or *rpb1-502* haploid strains. Evidently, this intermediate level of wild-type *HIS4* transcript is not sufficient to produce a His$^+$ phenotype. S1 nuclease analysis shows that the elevated transcript identified in the Northern blot of suppressed *rpb1* mutants initiates at or near the normal *HIS4* start site (Fig. 4).

The *rpb1-501* and *rpb1-502* mutations produce similar alterations in *lys2-128δ* transcription (data not shown). The predominant *LYS2* transcript in a *lys2-128δ* strain with wild-type RNA polymerase is approximately 600 nucleotides long (Fig. 1). This transcript is probably initiated at its normal site but is terminated prematurely at a site within the δ-element (18). *lys2-128δ* strains carrying either of the *rpb1* mutations produce, in addition to the transcripts found in an isogenic wild-type *lys2-128δ* strain, a *LYS2* RNA species which is approximately the same size as that observed in a wild-type *LYS2* strain. Since these strains are Lys$^+$, we infer that this new *LYS2* transcript is functional. Northern analysis demonstrates that the strong *spt* mutations in *RPB2* (*rpb2-503*, *rpb2-504*, and *rpb2-505*) produce transcriptional alterations at both *HIS4* (Fig. 5) and *LYS2* (data not shown) that are similar to those produced by the *rpb1* mutations.

Ty1 elements have, in addition to the major δ-initiated transcript, a minor transcript which initiates about 800 bp downstream (24, 74). Several class 1 *spt* mutations have been shown previously to selectively decrease the level of the major δ-initiated Ty1 transcript (73, 74). In contrast, our *rpb1* and *rpb2* mutations do not alter the ratio of the two Ty1 transcripts (data not shown).

The *rpb1* and *rpb2* mutations alter amino acids that are in close proximity. Both the *rpb1* and *rpb2* mutations are located near each other and may, therefore, identify regions of RNA polymerase II that affect transcription initiation. All of the mutations are single C-T transitions, resulting in single-amino-acid substitutions. The serine and threonine residues altered by the *rpb1-501* and *rpb1-502* mutations, respectively, are separated by only 15 amino acids, and both

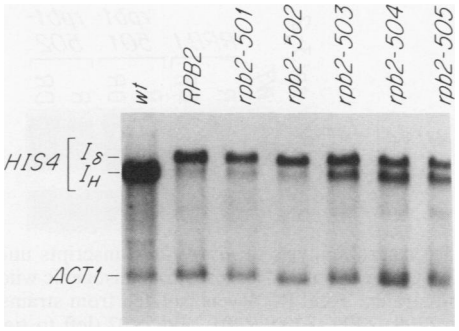

FIG. 5. Northern analysis of *his4-912δ* transcripts in isogenic wild-type (wt) and *rpb2* mutant strains. Total RNA isolated from strains Z27 and Z225 to Z230 (left to right) was subjected to denaturing gel electrophoresis and transferred to nitrocellulose. The Northern blot was probed with ^{32}P-labelled *HIS4* and *ACT1* riboprobes derived from pBC2 and pDH35, respectively.

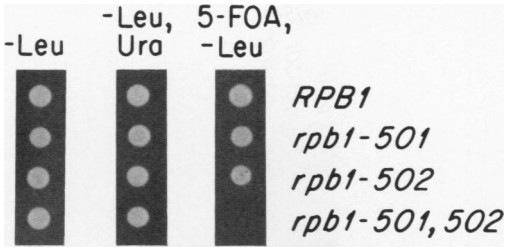

FIG. 7. An *rpb1-501 rpb1-502* strain is inviable. Isogenic strains Z456 to Z459 (top to bottom) were grown on SC lacking Leu and spot tested to SC lacking Leu, lacking Leu and Ura, and lacking Leu but having 5-FOA. Plates were incubated at room temperature for either 3 or (5-FOA plates) 5 days.

lie in a conserved region of RPB1 (Fig. 6A). This region, denoted homology block G, is the seventh of eight stretches of amino acids with extensive sequence similarity in the largest subunits of all three eucaryotic RNA polymerases and in the β′ subunit of *E. coli* RNA polymerase (for a review, see reference 20). The three strong semidominant *rpb2* alleles affect adjacent amino acids (Fig. 6B). In contrast to the *spt* mutations in *RPB1*, the strong mutations in *RPB2* do not occur in a homology block but are located between homology blocks B and C. The observation that *spt* mutations map in close proximity to each other in both *RPB1* and *RPB2* is likely to be significant. All 18 conditional lethal point mutations in *RPB1* and *RPB2* that have been derived from the same mutagenized plasmid libraries used in this study map outside of the regions defined by the *spt* mutations (56), and none suppresses the δ-insertion mutations (data not shown).

As stated previously, cell growth rate is not dramatically affected by single *spt* mutations in either *RPB1* or *RPB2*. However, strains carrying both *spt*-like *rpb1* mutations in the same gene are inviable (Fig. 7). Western analysis (see Materials and Methods) demonstrates that the double-mutant RPB1 subunit is present at a wild-type level (data not shown).

The *rpb1* mutations increase the 5′-end heterogeneity of *HIS4* transcripts. Although single *spt* mutations in *RPB1* do not have substantial effects on cell growth, it seemed possible that they would cause small, but detectable, changes in the pattern of transcript starts at some normal promoters. Indeed, both *rpb1* mutations produce a modest increase in the heterogeneity of transcription initiation at a wild-type

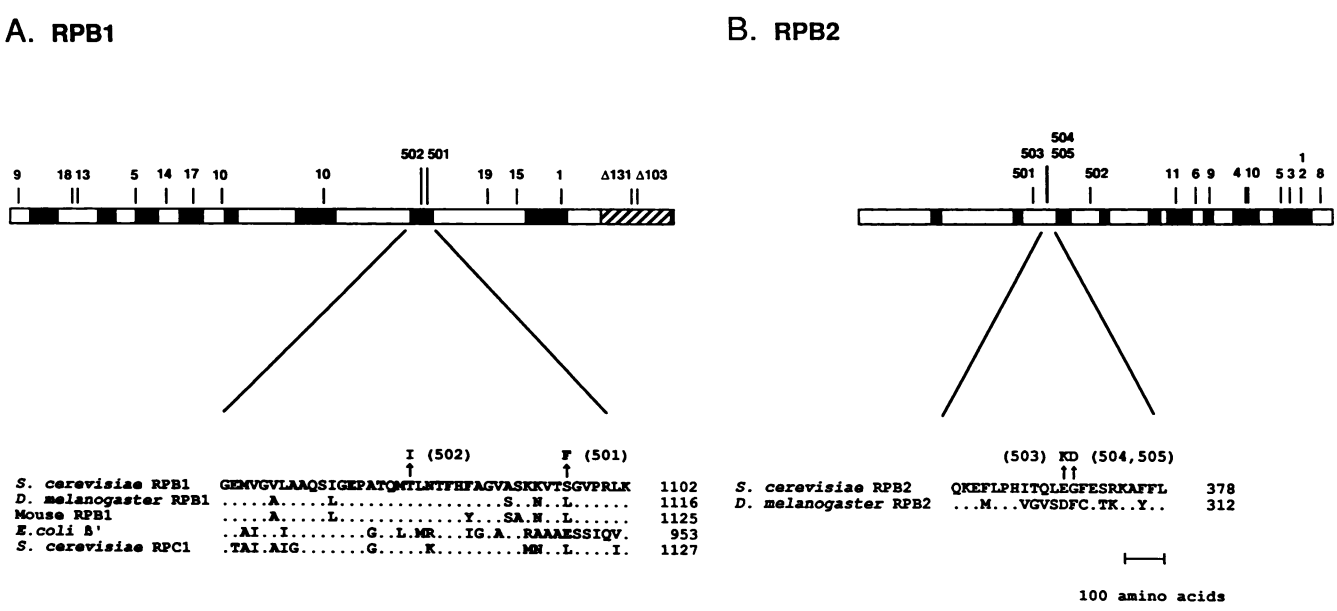

FIG. 6. Map positions of *rpb1* and *rpb2* mutations. The map positions of our *spt* mutations in *RPB1* and *RPB2* and the amino acid changes which would result from mutations *rpb1-501* and *rpb1-502* (A) or mutations *rpb2-503* to *rpb2-505* (B) are indicated. Both *rpb1* mutations alter residues within one of eight highly conserved stretches of amino acids, denoted homology block G (20). Mutations *rpb2-501* and *rpb2-502* produce a C-to-Y change at position 316 and an S-to-L change at position 488, respectively. The amino acid sequences in these regions are compiled from the data of references 2, 20, 25, and 66. Regions of RPB1 and RPB2 which are conserved with procaryotic β′ and β, respectively, are boxed; the carboxy-terminal repeat domain of RPB1 is striated. Map positions of conditional lethal *rpb1* and *rpb2* mutations, derived from the same mutagenized plasmid libraries, which do not suppress δ-insertion mutations, are from the data of Scafe et al. (56). The temperature-sensitive *rpb1-1* allele and the C-terminal truncation alleles *rpb1Δ101* and *rpb1Δ103* have been described previously (48, 49).

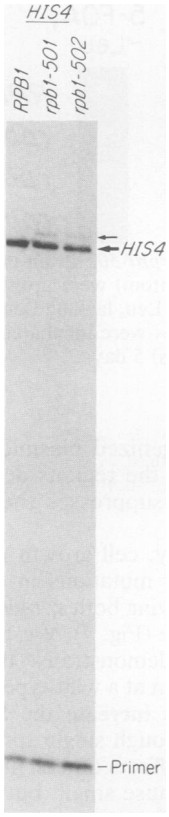

FIG. 8. Primer extension analysis of *HIS4* transcripts in isogenic wild-type and *rpb1* mutant strains. Total RNA from strains Z450, Z451, and Z452 (left to right) was hybridized to a ^{32}P-labelled oligonucleotide primer homologous to the *HIS4* early coding region. Hybrids were extended with reverse transcriptase, and the DNA extension products were resolved on a 6% polyacrylamide–urea sequencing gel. The shadow band 2 to 3 nucleotides longer than the major *HIS4* band, which is slightly more pronounced in lanes 2 and 3, can be resolved more clearly under different separation conditions.

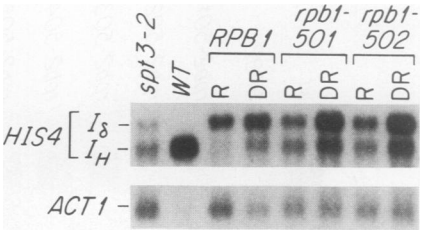

FIG. 9. Northern analysis of *his4-912δ* transcripts under conditions of GCN4 repression and derepression in isogenic wild-type and *rpb1* mutant strains. Total RNA was isolated from strains 8930-2C, Z27, Z197, Z198, Z199, Z200, Z201, and Z202 (left to right) grown under conditions of GCN4 repression (R) or derepression (DR), as indicated. RNAs were subjected to denaturing gel electrophoresis and transferred to nitrocellulose. The Northern blot was probed with ^{32}P-labelled *HIS4* and *ACT1* riboprobes derived from pBC2 and pDH35, respectively. The WT lane contains 1/10th the amount of RNA relative to that of the other lanes.

HIS4 promoter (Fig. 8). The level of at least one minor *HIS4* transcript, initiated at 7 to 10 nucleotides upstream of the major start site, was elevated in the *rpb1* mutant strains relative to an isogenic wild-type strain. Thus, the *rpb1* mutations perturb mRNA start site selection at at least one "normal" promoter. The patterns of transcript starts for two genes, *CYC1* and *CYH2*, do not appear to be substantially altered in the *rpb1* mutant strains, although in both cases a small increase is observed in the level of one of the minor transcripts initiated immediately upstream of the major start site (data not shown). Control experiments reveal that both the stability of RPB1 and nonspecific RNA polymerase II elongation activity in vitro are normal in extracts prepared from *rpb1* mutant strains (data not shown).

Mutant RNA polymerases remain UAS responsive. Unlike the previously reported *sit* (suppressor of initiation of transcription) mutations in *RPB1* and *RPB2* (4), the *spt* mutations in these genes do not appear to circumvent RNA polymerase II's dependence on UAS-responsive transactivators. The *sit* mutations suppress the His$^-$ phenotype of a strain lacking the GCN4, BAS1, and BAS2 transactivators of *HIS4* expression (4). In addition, the *sit1* and *sit2* mutations reduce cell growth in the absence of exogenous inositol as a result of an impaired ability to induce the *INO1* gene. The observations that neither of our *rpb1* mutations suppresses the His$^-$ phenotype of a "triple deletion" (*HIS4 gcn4 bas1 bas2*) strain nor produces an Ino$^-$ phenotype and that, conversely, representative *sit1* and *sit2* alleles of *RPB1* and *RPB2* fail to suppress δ-insertions (data not shown) suggest that the *spt* and *sit* mutations in *RPB1* and *RPB2* affect nonoverlapping functions.

his4-912δ transcription appears normally responsive to UAS-dependent transactivators in strains carrying the *rpb1* mutations. Both the δ- and *HIS4*-initiated *his4-912δ* transcripts have been shown previously to be responsive to derepression by GCN4 (61). This induction is also observed in *rpb1-501* or *-502* strains (Fig. 9). In all of the strains, both *his4-912δ* transcripts are induced approximately threefold. Furthermore, a genetic assay reveals that high-level production of the functional *his4-912δ* transcript requires, in addition to GCN4, the BAS1 and BAS2 transactivators (3) that are responsible for basal-level *HIS4* transcription (data not shown).

DISCUSSION

We have used a molecular genetic approach to identify mutations in the genes encoding the two large subunits of *S. cerevisiae* RNA polymerase II, *RPB1* and *RPB2*, that influence transcription initiation at specific promoters. The *spt* screen used to identify RNA polymerase II mutations affecting transcription initiation is exquisitely sensitive, allowing the detection of alterations that are sufficiently subtle to permit cell survival. Analysis of patterns of transcript starts in the *rpb1* mutant strains has provided evidence that eucaryotic RNA polymerase II, like its procaryotic counterpart (29, 52), contributes to accurate mRNA start site selection. Furthermore, the locations of the *rpb1* and *rpb2* mutations implicate specific regions of the subunits in transcription initiation.

The *spt* mutations in *RPB1* not only alter patterns of transcription initiation at δ-insertion promoters, but they also cause a small increase in the 5′-end heterogeneity of wild-type *HIS4* transcripts. Most likely, a similar effect occurs to various degrees at other *S. cerevisiae* promoters. This result indicates that RNA polymerase II, under constraints imposed by its association with other components of the initiation complex, can influence the precise site at which a transcript is initiated. In *E. coli*, the site of transcription

initiation is determined primarily through the interaction of RNA polymerase's σ subunit with conserved promoter sequences and secondarily through the purine nucleotide triphosphate preference of the active site of the enzyme (28, 29, 52, 60, 82). Recent cross-linking experiments with *E. coli* RNA polymerase have indicated that a segment of β′ which includes homology block G contacts the nascent transcript and, consequently, is located within the catalytic center of the polymerase (10). By analogy, the *spt* mutations in homology block G of RPB1 may affect the structure of the enzyme's active site. RNA polymerase II may normally scan a small region of the promoter for a thermodynamically favorable start site, under constraints imposed by its association with other components of the initiation complex, and alterations of the polymerase's catalytic site could perturb this process. It is possible that the *rpb1* mutations also affect a late component of the initiation pathway, such as promoter clearance.

The RNA polymerase II mutations described here represent another example of *spt* mutations that affect known components of the transcription initiation apparatus; the *SPT15* gene encodes the TATA-binding factor TFIID (23). Quite possibly, other class 1 and 2 *spt* mutations, which produce effects similar to those observed with the *spt15* and *rpb* mutations, occur in genes which encode additional components of the basic transcriptional machinery. The molecular basis for altered patterns of transcription initiation at δ-insertion promoters produced by *spt* mutations is not clear. Genetic evidence suggests that there exists a competition at *his4-912*δ between transcription initiation events at the δ and *HIS4* promoters (35). Hence, either an increase in the inherent efficiency of transcription initiation at the *HIS4* mRNA start site or a slight decrease in the level of initiation at the δ912 start site could produce a significant amplification of the level of *HIS4*-initiated *his4-912*δ transcript. Since the *rpb1* mutations have no discernible effect on the level of message initiated from a wild-type *HIS4* or 912δ promoter, a precise mechanism for the altered pattern of transcription initiation is unclear. It is possible that the changes in transcription initiation patterns observed at δ-insertion promoters result indirectly from perturbations of the enzyme's active site. Another possibility is that the mutations influence the interaction of RNA polymerase II with a general component of the TATA-associated transcriptional machinery which could, in turn, influence the approximate transcriptional start site.

With the *spt* mutations reported here, mutations in RNA polymerase II that affect three different aspects of transcription initiation have been identified. The *sit* mutations in *RPB1* and *RPB2* permit increased transcription of *HIS4* in the absence of the *trans*-acting factors that act at that promoter (4). Truncations of the carboxy-terminal repeat domain of RPB1 reduce the ability of RNA polymerase II to respond to signals from certain UAS elements (1, 55). The *spt* mutations in *RPB1* influence accurate initiation of mRNA synthesis but do not affect the normal UAS dependence of the polymerase. The *spt* mutants thus provide new tools to investigate specific aspects of transcription initiation.

ACKNOWLEDGMENTS

We are grateful to G. R. Fink for support, dialogue, and reagents; M. Nonet for plasmids and deletion strains; C. Scafe for hydroxylamine-mutagenized *RPB1* and *RPB2* DNA libraries; and F. Winston for strains and plasmids. We thank S. Buratowski, E. Elion, M. Nonet, D. Pellman, C. Scafe, and F. Winston for numerous stimulating discussions. S. Buratowski, G. R. Fink, P. Kolodziej, D. Pellman, C. Scafe, and F. Winston provided helpful comments on the manuscript. M. Rocha of Arrco Medical Art and Design expertly prepared many of the figures.

D.S.H. was the recipient of a National Science Foundation predoctoral fellowship. R.A.Y. is a Burroughs Wellcome Scholar. This work was supported by National Institute of Health grants GM 34365 to R.A.Y. and GM 40266 to G. R. Fink.

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