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RNA Polymerase II Subunit RPB3 Is an Essential Component of the mRNA Transcription Apparatus

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To improve our understanding of RNA polymerase II, the gene that encodes its third-largest subunit, *RPB3*, was isolated from a λgt11 DNA library by using antibody probes. The *RPB3* DNA sequence predicts a 318-amino-acid protein whose sequence was confirmed, in part, by microsequence analysis of the gel-purified RNA polymerase II subunit. *RPB3* was found to be an essential single-copy gene that is tightly linked to *HIS6* on chromosome IX. An *RPB3* temperature-sensitive mutant that arrested growth after three to four generations at the restrictive temperature was isolated. When the mutant was shifted to the restrictive temperature, RNA polymerase II could no longer assemble, previously assembled functional enzyme was depleted, and mRNA levels were consequently reduced. These results demonstrate that *RPB3* is an essential component of the mRNA transcription apparatus. Finally, the RPB3 protein is similar in sequence and length to RPC5, a subunit common to RNA polymerases I and III, suggesting that these subunits may play similar roles in RNA polymerases I, II, and III.

Eucaryotic RNA polymerases I, II, and III synthesize large rRNAs, pre-mRNAs, and small rRNAs and tRNAs, respectively (26, 35, 36). The three RNA polymerases are defined by the components that copurify with transcriptional activity in nonspecific elongation assays. They appear to be composed of 9 to 14 polypeptides whose molecular mass ranges from 10 to 240 kilodaltons (kDa). The subunit architectures and antigenicities of these RNA polymerases are well conserved among eucaryotes. The primary structures, relative stoichiometries, and transcriptional functions of eucaryotic RNA polymerase subunits are ill characterized.

The *Escherichia coli* RNA polymerase has been well defined structurally, and specific functions have been attributed to each of its subunits (reviewed in references 11 and 43). The procaryotic enzyme is composed of four different polypeptides, α (*rpoA*), β (*rpoB*), β′ (*rpoC*), and σ (*rpoD*). The sequences of the *rpo* genes indicate that α, β, β′, and σ are 36,511, 150,543, 155,163 and 70,263 Da, respectively. The functions of the subunits have been deduced by studying DNA-binding activity, susceptibility to affinity labeling with substrates, the ability to bind other proteins during enzyme reconstitution, and mutant phenotypes. These experiments indicate that the β′ subunit is largely responsible for DNA binding and that β binds ribonucleoside triphosphates and the RNA product. Both β′ and β contain portions of the catalytic site for RNA synthesis. Analysis of RNA polymerase mutants indicates that the α, β, and β′ subunits all contribute to the fidelity of transcription. Finally, mutant studies and reconstitution experiments suggest that the α subunit plays an important role in enzyme assembly.

The eucaryotic RNA polymerases have components that are structurally and functionally related to procaryotic RNA polymerase subunits. The largest subunits of RNA polymerases I, II, and III are homologs of the procaryotic β′ subunit (1, 2, 5, 31). The second-largest subunit of RNA polymerase II is homologous to β (16, 38) and is related antigenically to counterparts in RNA polymerases I and III (36). The smaller components of these eucaryotic enzymes remain ill defined. We report here the isolation of the gene for the third-largest subunit of yeast RNA polymerase II. This subunit is not similar to any of the procaryotic RNA polymerase subunits but is related to a subunit shared by RNA polymerases I and III and is an essential component of the mRNA transcription apparatus.

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MATERIALS AND METHODS

Materials and media. ^{32}P-labeled nucleotides and [^{35}S]-methionine were purchased from Amersham Corp., sequencing kits and exonuclease III were from New England BioLabs, Inc., terminal transferase and exonuclease VII were from Bethesda Research Laboratories, Inc., nick translation kits were from New England BioLabs or U.S. Biochemical Corp., RNases and protease inhibitors were from Sigma Chemical Co., restriction enzymes were from Promega Biotec, protein A-agarose was from Repligen, nitrocellulose was from Schleicher & Schuell, Inc., and Genescreen Plus was from DuPont, NEN Research Products. Orthogonal field alternation gel electrophoresis was done on a Beckman GeneLine apparatus. Yeast media were prepared as described by Nonet et al. (33). The 12CA5 monoclonal antibody was a generous gift of Gail Rogers and Ian Wilson.

***RPB3* sequence analysis.** The 2.9-kilobase (kb) *Pst*I DNA fragment from Y3097 was subcloned in both orientations into M13mp19, and partial deletions were isolated (41). Dideoxy sequencing was performed on both strands with a New England BioLabs kit. Computer analysis was performed by using either the NBRF or UWWCG program. The program tfasta, which searches all six reading frames of GENBANK, was used to identify sequence similarity to RPC5. The programs IALIGN and ALNED were used to maximize homology within parameters set by the Dayhoff mutation data matrix (14). The sequence has been entered into GENBANK under accession no. M27496.

Plasmids. The *URA3 CEN RPB3* plasmid pY2413 was constructed as follows. The 2.9-kb *Pst*I DNA fragment from Y3097 was cloned into the M13mp19 polylinker such that *RPB3* and *lacZ* were in the same transcriptional orientation

TABLE 1. Yeast strains[a]

Name	Genotype	Alias	Source
Z112	*MATα ura3-52 leu2-3,112 his3-Δ200 lys2-Δ201 ade2*	N222	This laboratory
Z113	*MATa ura3-52 leu2-3,112 his3-Δ200 lys2-Δ201 trp1Δ1*	N223	This laboratory
Z114	Z112 × Z113	K2	This laboratory
Z240	*MATa ura3-52 leu2-3,112 HIS4-912δ lys2-128δ*	H767	This laboratory
Z241	*MATα ura3-52 leu2-3,112 his3-Δ200 lys2-Δ201 ade2 RPB3* *MATa ura3-52 leu2-3,112 his3-Δ200 lys2-Δ201 trp1Δ1 rpb3Δ1::LYS2*	K8	This laboratory
Z242	*MATa ura3-52 leu2-3,112 HIS4-912δ lys2-128δ rpb3Δ1::LYS2* (pY2413)	K23	This laboratory
Z243	*MATα ura3-52 leu2-3,112 his6*	L855	A. Hinnebusch
Z244	*MATα ura3-52 leu2-3,112 his6 RPB3::3'LEU2*	K62	This laboratory
Z245	*MATa ura3-52 leu2-3,112 lys2-Δ201 cdc29-1*	K56	This laboratory
Z246	*MATα ura3-52 leu2-3,112 his3-Δ200 lys2-Δ201 ade2 RPB3::3'LEU2*	K57	This laboratory
Z247	*MATa ura3-52 leu2-3,112 his3-Δ200 lys2-Δ201 ade2 rpb3Δ1::LYS2* (pY2431)	K86	This laboratory
Z248	*MATa ura3-52 leu2-3,112 his3-Δ200 lys2-Δ201 ade2 rpb3Δ1::LYS2* (pY2432)	K87	This laboratory
Z249	*MATa ura3-52 leu2-3,112 HIS4-912δ lys2-128δ rpb3Δ1::LYS2* (pY2435)	K128	This laboratory
Z250	*MATa ura3-52 leu2-3,112 HIS4-912δ lys2-128δ rpb3Δ1::LYS2* (pY2437)	K129	This laboratory

[a] The notation *::3'LEU2* denotes an insertion of *LEU2* 3' to *RPB3* coding sequences. pY2435 and pY2437 are *RPB3 CEN* plasmids derived in part from pY2431 and bearing wild-type and *rpb3-1* alleles modified with the addition of the 12CA5 influenza virus epitope.

to produce pY2412. The 2.9-kb *Bam*HI-*Sph*I DNA fragment of pY2412 was inserted into YCp50 to produce pY2413.

Plasmid pY2433 is a pBR322-based plasmid that contains a precise replacement of *RPB3* coding sequences with *LYS2*. The deletion *rpb3Δ1* was constructed by using the *dut ung* mutation site-directed mutagenesis method (23) with the oligonucleotide CAATATTGAAAAATGAATTCTAGACC CGGGCGTAATAGAGGAAA (underlined sequences are immediately 5' and 3' to the coding region) and pY2412 single-stranded DNA. This sequence replaces the *RPB3* coding region (nucleotides 493 to 1451; see Fig. 2) with an *Eco*RI-*Xba*I-*Sma*I polylinker. The *rpb3Δ1* allele was subcloned as a 2-kb *Bam*HI-*Sph*I DNA fragment into pFBI14 (3), generating pY2417. The 6.4-kb *Sma*I-*Cla*I fragment from pDA6200 (4) containing the *LYS2* gene was blunted with Klenow fragment and inserted into the *Sma*I site of pY2417 to produce pY2433. The transcriptional orientation of *LYS2* is opposite that of *RPB3*.

The *LEU2 CEN RPB3* plasmid pY2431 was constructed in several steps. The Y3097 2.9-kb *Pst*I DNA fragment was inserted into pFBI14 (3) to make pY2404. The 1-kb *Sal*I-*Hin*dIII DNA fragment containing most of the *RPB3* coding region from pY2404 was inserted into pBR322 to produce pY2416. The 1-kb *Sal*I-*Eco*RI DNA fragment of pY2416 was cloned into pY2417 to produce pY2420. Plasmid pY2421 was created by inserting the 1.4-kb *Sal*I-*Sph*I DNA fragment of pY2420 into pBR322. The 2.2-kb *Xba*I-*Sma*I DNA fragment of pY2408, containing the *LEU2* gene, was inserted into the polylinker of pY2421, forming pY2423. *LEU2* and *RPB3* are transcribed in opposite directions in pY2423. Plasmid pY2408 has a 2.2-kb *Sal*I-*Xho*I DNA fragment containing *LEU2* inserted into the *Bam*HI site of pUC18 by partial fill-in with reverse transcriptase. The 3.6-kb *Sal*I-*Sph*I DNA fragment of pY2423 was inserted into pY2425 to produce pY2427. Plasmid pY2425 was constructed by inserting the 1.7-kb *Bgl*II-*Sph*I DNA fragment of pY2403 into pFBI14. Plasmid pY2403 contains the 2.9-kb *Eco*RI-*Sph*I fragment of pY2412 inserted into pBR322. The 3.9-kb *Bgl*II-*Sph*I DNA fragment of pY2427 was inserted into Y2403 to make pY2429. Finally, pY2431 was constructed by moving the 5.1-kb *Bam*HI-*Sph*I DNA fragment bearing the *LEU2 RPB3* cassette from pY2429 into pY2401. Plasmid pY2401 is the product of ligating the 5.4-kb *Xmn*I DNA fragment containing *CEN* and *ARS* from YCp50 with the 2.4-kb *Xmn*I DNA fragment of pBR322.

To create pY2435, sequences encoding an epitope from the influenza virus hemagglutinin protein was added to the amino terminus of RPB3 by oligonucleotide-mediated site-directed mutagenesis of pY2434. Plasmid pY2434 carries the 5.1-kb *Bam*HI-*Sph*I *LEU2 RPB3* cassette of pY2431 inserted into the pUC18 polylinker present in pY2436. Plasmid pY2436 is a *CEN ARS* plasmid with an M13 origin embedded between *CEN* and *ARS*. To construct pY2436, the 2.5-kb *Nar*I-*Hin*dIII DNA fragment of pUC18 containing the polylinker was ligated to the 1.9-kb *Nar*I-*Hin*dIII fragment of pUN70 bearing the *CEN* M13 *ARS* functions (15). To produce pY2437, the *rpb3-1* allele was tagged by replacing the 3.6-kb *Sal*I-*Sph*I DNA fragment of pY2435 with the equivalent pY2432 fragment. Plasmid pY2432 was generated from pY2431 by hydroxylamine mutagenesis.

Yeast strains. The *Saccharomyces cerevisiae* strains used are listed in Table 1. Strains Z112 and Z113 and derivatives descend from S288C strains originally obtained from D. Botstein. Z241 was derived from Z114 by homologous integration of the 8.4-kb *Pst*I DNA fragment from pY2433. Z242 was obtained by transforming Z240 first with pY2413 and then with the 8.4-kb *Pst*I DNA fragment of pY2433. The *RPB3::3'LEU2* gene cassette from pY2431 was integrated into Z243 to make Z244 and into Z112 to generate Z246. Z245 was obtained from a cross between Z112 and strain 4864-1D (*MATa cdc29-1*), obtained from G. Fink. Z247 and Z248 were obtained by sporulating Z241 after transformation with pY2413 and then replacing pY2413 with pY2431 (in Z247) or pY2432 (in Z248). Z249 and Z250 were derived from Z242 by replacing pY2413 with pY2435 (in Z249) or pY2437 (in Z250). Yeast cells were transformed by the lithium acetate method (24).

Isolation and sequence analysis of *rpb3-1*. Plasmid pY2431 DNA (20 μg/ml) was treated with 1.0 M hydroxylamine hydrochloride (pH 6.8)–1 mM EDTA for 30 h at 37°C. The mutagenized plasmids were used to transform Z242, and 5,000 individual colonies were picked and arrayed on DO-lys, leu plates (33), replica plated to 5-fluoro-orotic acid, DO-lys, leu plates, and transferred to yeast extract-peptone-dextrose medium at 12, 24, and 38°C. The frequency of inviable alleles of *RPB3* (5-fluoro-orotic acid-sensitive colonies at 24°C out of all colonies) was between 1 and 2%. Plasmids were isolated from temperature-sensitive colonies by glass bead disruption, phenol extraction, and ethanol precipitation, followed by transformation of *E. coli* and then

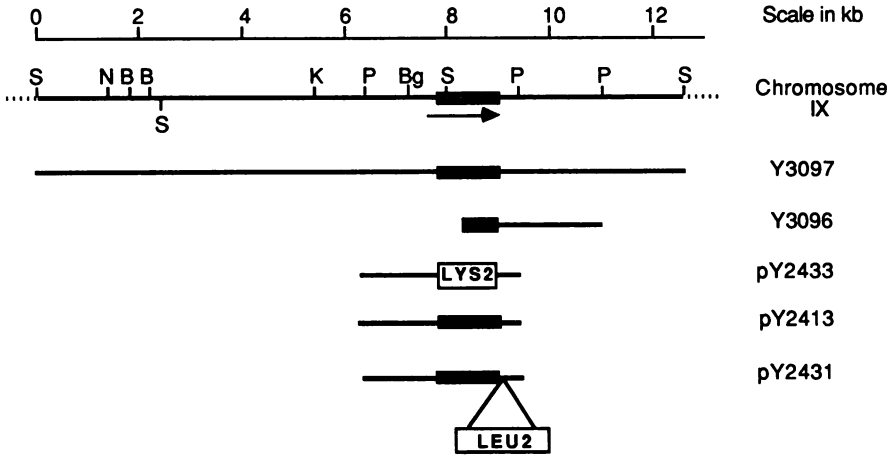

FIG. 1. Restriction map of the *RPB3* locus on chromosome IX. Symbols: ▨, *RPB3* open reading frame; →, *RPB3* mRNA. Y3097 is an EMBL3 clone, and Y3096 is a λgt11 clone. Plasmid pY2433 contains DNA from the *RPB3* locus in which the *RPB3* coding region is replaced with the *LYS2* gene, pY2413 is a *URA3 CEN* plasmid that contains *RPB3*, and pY2431 is a *CEN* plasmid that contains *RPB3* and *LEU2*. Abbreviations for restriction sites: B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; N, *Nru*I; P, *Pst*I; S, *Sal*I.

yeast strain Z242. One candidate mutant allele, *rpb3-1*, produced temperature-sensitive cells after a plasmid shuffle in Z242. Supercoiled plasmid DNA carrying *rpb3-1* was sequenced with oligonucleotide primers as described by Chen and Seeburg (12).

Northern (RNA) analysis. Yeast strains Z247 and Z248 were grown to saturation at 24°C in yeast extract-peptone-dextrose medium. Parallel cultures at either 24 or 38°C were inoculated from these cultures, and cells were grown to an optical density at 600 nm of 0.1 to 1. Equivalent cell numbers were harvested by centrifugation, and RNA was prepared by glass bead disruption and serial phenol extraction. RNA concentrations were normalized by optical density at 260 nm and by rRNA content (by staining the nitrocellulose with methylene blue [27]). Samples containing equal amounts of the large rRNAs were subjected to electrophoresis in 1% formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized with nick-translated DNA probes (27, 29). The plasmids that were used as probes were pY2085 (a pUC18 plasmid containing a 7.1-kb *Hin*dIII DNA fragment of *RPB1* [42]), a pY2315 (a pGEM3 plasmid containing a 5.3-kb *Eco*RI-*Hin*dIII DNA fragment bearing *LYS2* [4]), pY2317 (a pGEM3 plasmid containing a 1.65-kb *Bam*HI-*Hin*dIII DNA fragment bearing *ACT1* [32]), pY2384 (a pUC8 plasmid containing a 2.6-kb *Xho*I-*Sal*I DNA fragment bearing *DED1* [37]), and pYG100 (a pBR322 plasmid bearing the 8-kb *Bam*HI-*Hin*dIII DNA fragment bearing *SSA1* [20]). After hybridization, the blots were washed four times at 55°C in 0.1× SSPE (27)–0.1% sodium dodecyl sulfate (SDS).

Immunoprecipitation of epitope-tagged *RPB3* and *rpb3-1* protein. Cultures of 25 ml at 24°C were inoculated from overnight cultures to a starting optical density at 600 nm of 0.3. The culture medium was yeast nitrogen base-glucose supplemented with lysine, leucine, histidine, and uracil. After 2 h of growth, the cultures were either shifted to 38°C for 30 min or allowed to continue at 24°C. [^{35}S]methionine (25 μCi/ml) was added, and growth continued for 2 h. These cultures produced the samples shown in lanes 1 to 6 of Fig. 5. Two additional cultures, one of Z249 and one of Z250, were labeled after 1 h of growth at 24°C for 2 h. After labeling, these cells were pelleted and suspended in fresh medium containing 10 mM methionine, allowed to grow at 24°C for 30 min, and then shifted to 38°C for 1 h.

Cells were harvested by centrifugation, washed once with cold distilled water, suspended in lysis buffer at 4°C, and disrupted by vortexing with glass beads. All subsequent steps were performed at 4°C. Cells were lysed in buffer containing 20 mM *N*,*N*-hydroxyethylpiperazine-*N*,*N*'-ethanesulfonic acid (HEPES) NaOH (pH 7.9), 0.35 M $(NH_4)_2SO_4$, 10% glycerol, 1 mM dithiothreitol, 5 mM benzamidine, and 10 μg of chymostatin, pepstatin, leupeptin, aprotinin, and antipain per ml. Lysates were fractionated with polymin P as described by Bitter (6). The $(NH_4)_2SO_4$ pellets from polymin P fractionation were suspended in immunoprecipitation buffer (IB) [20 mM HEPES NaOH (pH 7.9), 0.05 M $(NH_4)_2SO_4$, 10% glycerol, 1 mM dithiothreitol, 5 mM benzamidine, 2.5 mg of bovine serum albumin per ml, 10 μg of chymostatin, pepstatin, leupeptin, aprotinin, and antipain per ml, 1% Triton X-100, 0.1% SDS]. The samples were precleared by incubation with protein A-agarose and centrifugation, incubated with 12CA5 antibody for 2 h, and then incubated with protein A-agarose beads for 2 h. The beads were pelleted and washed twice with IBA (IB containing 0.1% Tween 20 instead of 1% Triton X-100 and 0.1% SDS) and once with IBA lacking bovine serum albumin; the samples were then boiled in Laemmli loading buffer (25). Samples were electrophoresed on 12.5% acrylamide (0.125% bisacrylamide) SDS-polyacrylamide gels, and fluorography was performed by soaking in 20% (wt/wt) 2,5-diphenyloxazole-acetic acid.

RESULTS

RNA polymerase subunit gene isolation. The *S. cerevisiae* *RPB3* gene was cloned by screening a λgt11 genomic DNA library (42) with a monoclonal antibody that recognizes the third-largest subunit (45 kDa) of yeast RNA polymerase II (39). Clone Y3096 (Fig. 1) produced an antibody-positive β-galactosidase fusion protein. The insert DNA of Y3096 was used to isolate a λEMBL3 yeast DNA clone, Y3097, that contained a larger genomic DNA insert. Southern analysis showed that the *RPB3* locus is single copy.

The amino acid sequence of the RPB3 protein was deduced from DNA sequence analysis of the 1.8-kb *Bgl*II-*Pst*I DNA fragment of Y3097 (Fig. 2). The *RPB3* sequence predicts a 318-amino-acid protein of 35,283 Da. Microse-

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AGATCTTGGAGTTTGTGCGCGGGGGTTCTAGACAATTCACCTGATTGGTTTCGATGTACTCTTTTGTTTTCTTTTCCTTTTCCTTCCTAT    90
TCCGCTTGTAAATAACTCCGTTATGTCTCACCTTCGATCCCCACTTTTTCTTTTGGAATACTGCATCAAATAGGAAAGAGAGAAAAAAGG   180
AAATCATATCCCAATATATTTGAACATACGACACGATATACCTTATATAGTATCATCTGTACTAATAGTACTATAACTAATACTTCTTGA   270
                                                                                          **
CGGACAAGCTTCGCAACTCGTTCGCAATGACCAAGTTTGGCTTTCGGGAAGCAAAGTGGAAAAAAAATGAAAAATAAACTAATTGAAGTGG   360
          *
TAAAAAATAAGCTGTGTGTGTGCATATACATCGTAAGTGCGTTGGCTGTTCTGTGGAAAAGGGAGACACTTTTTATATCAGGTGCAAAGG   450

CTTCGTACCAGCGTTGAAAAACTAAAACAATATTGAAAAATGAGTGAAGAAGGTCCTCAAGTTAAAATAAGAGAAGCTAGTAAAGATAAC   540
                                        MetSerGluGluGlyProGlnValLysIleArgGluAlaSerLysAspAsn
                                      A
GTCGACTTTATACTATCCAATGTGGATTTGGCTATGGCCAATTCTCTCCGTAGAGTCATGTAGCAGAGATTCCCACTTTAGCCATTGATA   630
ValAspPheIleLeuSerAsnValAspLeuAlaMetAlaAsnSerLeuArgArgValMetIleAlaGluIleProThrLeuAlaIleAsp
                                      Asp
TCAGTCGAAGTCGAAACAAATACTACGGTTTTGGCCGATGAATTTATTGCACACAGATTAGGTTTAATACCTCTACAAAGTATGGATATC   720
SerValGluValGluThrAsnThrThrValLeuAlaAspGluPheIleAlaHisArgLeuGlyLeuIleProLeuGlnSerMetAspIle

GAACAACTAGAATACAGTCGTGATTGTTTTTGTGAGGACCACTGTGATAAGTGTTCAGTGGTGTTAACATTGCAAGCGTTCGGCGAAAGC   810
GluGlnLeuGluTyrSerArgAspCysPheCysGluAspHisCysAspLysCysSerValValLeuThrLeuGlnAlaPheGlyGluSer

GAAAGTACTACAAACGTCTACAGCAAGGACTTGGTAATTGTGTCTAATCTCATGGGCCGTAATATTGGGCATCCTATTATACAAGATAAA   900
GluSerThrThrAsnValTyrSerLysAspLeuValIleValSerAsnLeuMetGlyArgAsnIleGlyHisProIleIleGlnAspLys

GAAGGTAACGGTGTTCTTATTTGTAAATTGAGAAAAGGTCAAGAATTGAAACTGACATGCGTTGCGAAAAAGGGTATAGCCAAAGAGCAC   990
GluGlyAsnGlyValLeuIleCysLysLeuArgLysGlyGlnGluLeuLysLeuThrCysValAlaLysLysGlyIleAlaLysGluHis
                        C
GCCAAGTGGGGTCCAGCAGCAGGCATAGAGTTCGAGTATGATCCTTGGAATAAGCTCAAACATACCGATTACTGGTATGAACAGGATTCT  1080
AlaLysTrpGlyProAlaAlaGlyIleGluPheGluTyrAspProTrpAsnLysLeuLysHisThrAspTyrTrpTyrGluGlnAspSer
                       Ala
GCCAAAGAATGGCCTCAGTCTAAAAATTGTGAATACGAAGATCCTCCAAACGAAGGTGACCCCTTCGATTATAAGGCTCAGGCAGATACA  1170
AlaLysGluTrpProGlnSerLysAsnCysGluTyrGluAspProProAsnGluGlyAspProPheAspTyrLysAlaGlnAlaAspThr

TTTTATATGAATGTAGAATCTGTGGGGTCTATTCCCGTTGATCAAGTTGTCGTCAGAGGTATCGATACATTACAGAAAAAGGTTGCCTCA  1260
PheTyrMetAsnValGluSerValGlySerIleProValAspGlnValValValArgGlyIleAspThrLeuGlnLysLysValAlaSer

ATATTGTTAGCTCTGACACAGATGGATCAAGACAAAGTTAATTTTGCATCGGGTGATAACAACACCGCTTCAAATATGCTGGGAAGTAAC  1350
IleLeuLeuAlaLeuThrGlnMetAspGlnAspLysValAsnPheAlaSerGlyAspAsnAsnThrAlaSerAsnMetLeuGlySerAsn

GAAGACGTAATGATGACGGGCGCTGAGCAAGATCCTTACTCCAATGCATCTCAAATGGGTAATACTGGATCAGGAGGGTATGATAATGCT  1440
GluAspValMetMetThrGlyAlaGluGlnAspProTyrSerAsnAlaSerGlnMetGlyAsnThrGlySerGlyGlyTyrAspAsnAla

TGGTAGTTCTCAAGTGGGCGTAATAGAGGAAAAAAAACAAGTGAACGAACCGAAAATAATAAGAGAAAAAAAGCTTTATATGCATGTATG  1530
TrpEND
TATCATTATGATTAGTATTATAAGTATTATAATCTAAAGTACGAAGCAAACAAAAAAGAAAAAAGAAAATGAAAAAATGAAAGGAAGGGC  1620
TAACCTTGCTTTTCATTCCATCTACAGCAGTCTTCAAACTTAACTAGGTTACCACCAAATATATCTAAGGAACTACCGAATGTCAAATCT  1710
ACTTTTCCGTGACTTAGTTCGTCTACTAATTTCAAATCATCAACACTTTTGGCCCCACCAGCATAAACGATTTTCAAATCATCGTAATCT  1800
TTGGTCCATTCGAAAAGCTTAGAAACCAATAATTCATCGATACCACCACACAAACCTTCAACGTCTGCAG                       1870
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FIG. 2. Nucleotide sequence of *RPB3* and amino acid sequence of the subunit. The 1,867-base-pair *Bgl*II-*Pst*I fragment containing this sequence complements the *rpb3Δ1* deletion. *, Start points of transcription ±1 as mapped by RNase protection (30). The two nucleotide changes in the *rpb3-1* mutant are shown above the sequence; the amino acid substitutions are italicized below the sequence. The RPB3 protein was gel purified from a yeast RNA polymerase II preparation and proteolyzed, and one of the fragments was sequenced; the 17-amino-acid sequence obtained is underlined.

quence analysis of a portion of the gel-purified 45-kDa RNA polymerase II subunit confirmed in part the predicted amino acid sequence (N. Woychik and R. Young, unpublished data). The *RPB3* mRNA start site was mapped by analyzing the RNase-resistant products of hybridization between a radiolabeled SP6 antisense transcript of *RPB3* and yeast poly(A)$^+$ RNA as described by Melton et al. (30). These results are summarized in Fig. 2.

The *RPB3* open reading frame is considerably smaller than that expected for the third-largest RNA polymerase II subunit, which has an apparent molecular mass of 45 kDa on SDS-polyacrylamide gels (36). However, when yeast mRNA was isolated by hybridization with *RPB3* DNA and then translated with a rabbit reticulocyte extract in vitro, the protein product migrated on SDS-polyacrylamide gels with an apparent molecular mass of 45 kDa (not shown). Thus, the aberrant mobility of the RPB3 protein on SDS-polyacrylamide gel electrophoresis is probably due to its amino acid composition or sequence.

Mapping of *RPB3*. Physical and genetic mapping revealed that *RPB3* is tightly linked to *HIS6* on chromosome IX. *RPB3* was first physically mapped to chromosome IX by nitrocellulose blot hybridization to electrophoretically separated yeast chromosomes (9). *RPB3* was further mapped by

meiotic methods and found to be tightly linked to *HIS6*. No recombination between *HIS6* and the *LEU2*-marked *RPB3* gene was observed among 107 tetrads dissected from a cross between Z244 and Z245. The map order with respect to *cdc29* could not be determined.

***RPB3* is essential for cell viability.** The single chromosomal *RPB3* gene was deleted to assess whether the gene is essential for yeast cell viability. One copy of *RPB3* was replaced with a *LYS2* gene by transforming the Lys$^-$ diploid Z114 with pY2433 (Fig. 1) and selecting for lysine prototrophs. Twenty tetrads were dissected from the resulting strain, Z241. Colonies grew from only two spores in each tetrad, and these were all Lys$^-$. The other two spores divided once or twice, suggesting that sufficient *RPB3* product was transmitted from the diploid for germination but not for continued vegetative growth.

Reduced mRNA levels in an *RPB3* conditional mutant. A temperature-sensitive *RPB3* mutant was isolated by using a plasmid shuffle scheme (8, 28). Strain Z242, which carries an *RPB3* chromosomal deletion that is complemented by a wild-type copy of *RPB3* on the *URA3 CEN* plasmid pY2413 (Fig. 1), was transformed with a library of hydroxylamine-mutagenized *LEU2 CEN RPB3* plasmids (pY2431). Leu$^+$ transformants were transferred to medium containing the drug 5-fluoro-orotic acid to select for cells that had lost the *URA3* plasmid bearing the wild-type *RPB3* gene (7). Five thousand Leu$^+$ Ura$^-$ transformants were screened for temperature-sensitive phenotypes, and one recessive mutant, *rpb3-1*, was isolated. Cells containing *rpb3-1* on centromere-containing plasmids grew at wild-type rates at 24°C but arrested growth after three to four divisions at 38°C. Cell viability declined approximately twofold after 16 h at 38°C. Sequence analysis of *rpb3-1* DNA revealed two mutations. One mutation produced an alanine-to-aspartic acid substitution, and the other produced a glycine-to-alanine change (Fig. 2).

Reduced levels of mRNAs were found at the restrictive temperature in *rpb3-1* mutants, confirming that the RPB3 protein is involved in mRNA synthesis (Fig. 3). RNA was isolated from *RPB3* wild-type and mutant cells before and after a shift to the restrictive temperature. The synthesis of rRNA and tRNA in *rpb3-1* mutant cells appeared to be relatively unaffected for up to 9 h after a temperature shift, since the yield of total RNA from cells changed little during this time. Samples containing equal amounts of the two large rRNAs were subjected to formaldehyde-gel electrophoresis, transferred to nitrocellulose, and hybridized with various gene probes. Three to four generations after the temperature shift (9 h), mutant cells had reduced levels of four of five mRNAs examined. The levels of *LYS2*, *DED1*, and *ACT1* mRNAs decreased, and no heat shock induction of *SSA1* mRNA was observed in the *rpb3-1* mutant. *RPB1* mRNA persisted at approximately wild-type levels. The *RPB1* mRNA levels probably remained constant because the message was relatively stable (33), and the *RPB1* promoter may compete efficiently for dwindling supplies of RNA polymerase II.

RNA polymerase II is not assembled in *rpb3-1* mutants at the nonpermissive temperature. The kinetics and pattern of mRNA depletion in the *RPB3* mutant suggested that the *rpb3-1* mutation produces a marginal defect in RNA polymerase II function or a defect in RNA polymerase II assembly. The half-lives of *DED1* and *RPB1* mRNAs are approximately 6 and 60 min, as measured in an RNA polymerase II mutant (*rpb1-1*) that ceases mRNA synthesis immediately after a temperature shift (33). The rate of

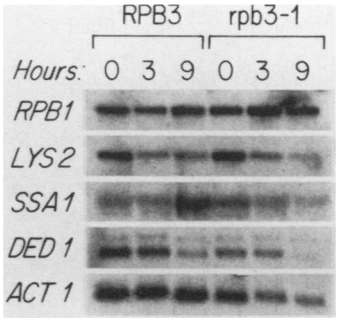

FIG. 3. Analysis of mRNA levels in *RPB3* wild-type (Z247) and *rpb3-1* mutant (Z248) strains. RNA was isolated from wild-type and mutant cells from cultures grown to mid-log phase at 24°C (0 h) and from cultures shifted to the nonpermissive temperature (38°C) for 3 or 9 h. RNA preparations obtained from a single temperature shift experiment and normalized in rRNA content were subjected to electrophoresis and immobilized on three separate nitrocellulose filters. Specific mRNAs were probed with DNA fragments containing *RPB1* (the largest subunit of RNA polymerase II), *LYS2* (α-aminoadipate reductase), *SSA1* (a 70-kDa heat shock protein), *DED1* (an essential gene adjacent to *HIS3*), and *ACT1* (actin). The first filter was probed with *RPB1*, *LYS2*, and *DED1*, the second was probed with *RPB1* and *SSA1*, and the third was probed with *RPB1* and *ACT1*. The *RPB1* data from the first filter are presented and are representative of results obtained for the three filters.

depletion of these two mRNAs at the restrictive temperature in the *rpb3-1* mutant was considerably slower. We investigated whether the *RPB3* mutant subunit affects assembly of RNA polymerase II at the nonpermissive temperature through immunoprecipitation of RPB3 and associated proteins.

To assay the ability of RPB3 to assemble into RNA polymerase II, subunit-specific immunoprecipitation of RPB3 protein was developed. The amino termini of wild-type and mutant RPB3 proteins were tagged with a nine-amino-acid sequence recognized by the anti-influenza virus hemagglutinin monoclonal antibody 12CA5 (17). The epitope-coding sequence was incorporated via oligonucleotide-mediated, site-directed mutagenesis and did not alter the growth properties of *RPB3* or *rpb3-1* cells. When immunoprecipitated from extracts of [^{35}S]methionine-labeled wild-type cells, the RPB3 subunit was associated with polypeptides that comigrated with previously defined RNA polymerase II subunits (Fig. 4). Because the numbers of methionine residues in the three largest subunits are 44 in RPB1, 38 in RPB2, and 11 in RPB3, the stoichiometry of the three subunits in the RNA polymerase II molecule could be calculated. These data indicate that the molar ratios of RPB1, RPB2, and RPB3 are 1:1:2.

An assembly defect was revealed in *rpb3-1* cells when radiolabeled RNA polymerase II was immunoprecipitated from cells pulse-labeled with [^{35}S]methionine before and after a temperature shift (Fig. 4). The pattern of RPB3-associated proteins was identical when RPB3 was immunoprecipitated from wild-type cells pulse-labeled at 24 and 38°C. The same proteins were associated with mutant RPB3 protein when immunoprecipitated from *rpb3-1* cells pulse-labeled at 24°C, but no proteins were associated with the mutant RPB3 subunit from *rpb3-1* cells pulse-labeled at 38°C. Thus, although the mutant RPB3 subunit accumulated to detectable levels in *rpb3-1* cells at the restrictive temperature, it did not associate with other RNA polymerase II subunits at this temperature. RNA polymerase II assembled

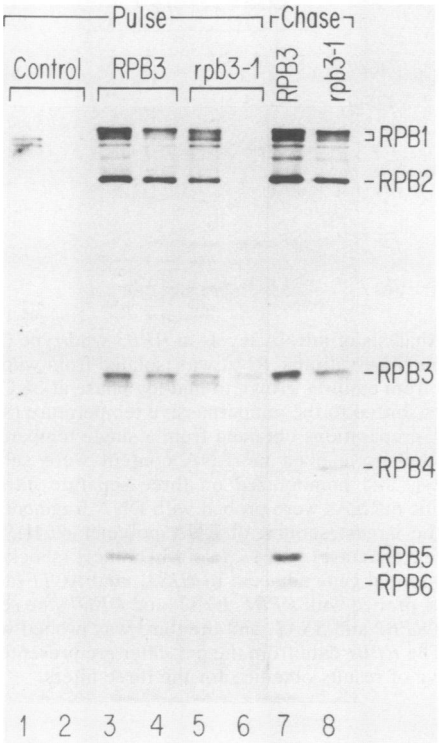

FIG. 4. RNA polymerase II levels in *RPB3* wild-type and mutant strains. RNA polymerase II subunits associated with the RPB3 protein were studied by preparing extracts from [^{35}S]methionine pulse-labeled cells, precipitating protein with polymin P, and then immunoprecipitating epitope-tagged RPB3 and associated proteins. The immunoprecipitate was subjected to 7.5% SDS-polyacrylamide gel electrophoresis; the radiolabeled proteins appear on the autoradiograph. Immunoprecipitated proteins are from wild-type cells in which RPB3 was not epitope tagged (Z242), labeled at 24°C (lane 1) and 38°C (lane 2); wild-type cells containing epitope-tagged RPB3 (Z249), labeled at 24°C (lane 3) and 38°C (lane 4); *rpb3-1* mutant cells containing epitope-tagged RPB3 (Z250), labeled at 24°C (lane 5) and 38°C (lane 6); wild-type cells containing epitope-tagged RPB3 (Z249), labeled at 24°C and chased with cold methionine for 1 h at 38°C (lane 7); and *rpb3-1* mutant cells containing epitope-tagged RPB3 (Z250), labeled at 24°C and chased with cold methionine for 1 h at 38°C (lane 8).

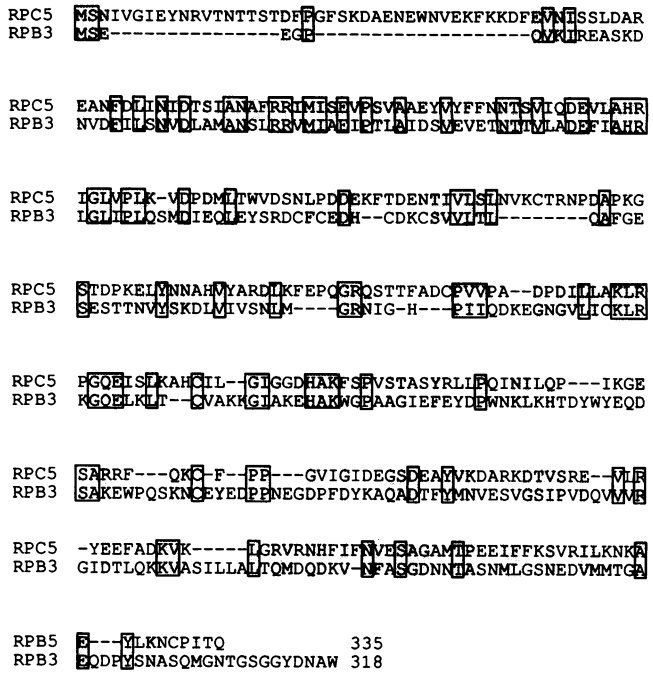

FIG. 5. Amino acid similarities between RNA polymerase subunits RPB3 and RPC5. Boxed residues are identical and shaded residues are similar, as assessed by the Dayhoff mutation data matrix. Sequences were aligned by using the program IALIGN (14).

in *rpb3-1* cells before the temperature shift could be immunoprecipitated after growth at 38°C, indicating that previously assembled RNA polymerase II is stable at the restrictive temperature. Taken together, these data explain the slow development of the transcriptional defects exhibited by the *rpb3-1* mutants. At the restrictive temperature, unequal competition among genes for a dwindling pool of assembled RNA polymerase II can account for the slow and unequal rates of depletion of the different mRNAs.

RPB3 protein is related to a subunit shared by RNA polymerases I and III. The RPB3 protein sequence was compared with protein sequences in the GENBANK data base. A yeast RNA polymerase subunit shared by RNA polymerases I and III (*RPC5*, alias *RPC40*) (28) is similar in sequence throughout the length of the RPB3 protein and is nearly identical to RPB3 in length (Fig. 5). We found that 31% of the amino acid residues of RPB3 and RPC5 are identical and that an additional 20% are conserved substitutions, as scored by the Dayhoff mutation data matrix. The yeast RPB3 and RPC5 subunits have counterparts in higher eucaryotes that are conserved in size and in antigenic cross-reactivity (36).

DISCUSSION

The definitive subunit composition of each of the eucaryotic nuclear RNA polymerases is not yet known, and the functions of each subunit are poorly understood. However, several of the components of yeast RNA polymerase II have now been defined through molecular and genetic analysis. Of 10 polypeptides that copurify with transcriptional activity, the 4 largest, RPB1 (192 kDa), RPB2 (139 kDa), RPB3 (35 kDa), and RPB4 (25 kDa), have now been shown to have roles in the mRNA transcription apparatus. RPB1 and RPB2 are homologs of the *E. coli* RNA polymerase β′ and β subunits (2, 38) and are essential for mRNA, but not rRNA or tRNA, synthesis (33; C. Scafe and R. A. Young, unpublished data). We have shown here that RPB3 is essential for cell viability and that *RPB3* mutants can disrupt RNA polymerase II assembly and mRNA biosynthesis. Although RPB4 is not essential for cell viability, it is essential for normal levels of mRNA synthesis; RNA polymerase II lacking RPB4 elongates less efficiently and is thermosensitive (40). RPB3 and RPB4 do not appear to be homologs of any of the other procaryotic RNA polymerase subunits. The genes that encode the four largest RNA polymerase II subunits are all single copy in the yeast haploid genome and reside on different chromosomes.

The sequence relatedness of RPB3 and RPC5 and similarities in their mutant phenotypes indicate that they may play similar roles in RNA polymerases I, II, and III. Analysis of yeast RNA polymerase II assembled before and after a shift to the nonpermissive temperature in *RPB3* mutant cells revealed that RNA polymerase II assembled before the shift

is relatively stable and functional, even at the restrictive temperature. RPB3 protein synthesized at the restrictive temperature does not associate with any other RNA polymerase II subunits. Thus, RNA polymerase II assembled before the temperature shift must be able to sustain growth of *RPB3* mutant cells for several generations at the restrictive temperature, and cessation of cell growth is probably due to enzyme dilution and degradation. Mutations in the related RPC5 subunit appear to affect RNA polymerase I and III assembly in a similar manner (28). These mutants cease growth only after several generations at the nonpermissive temperature, concomitant with reduced rRNA synthesis. In one *RPC5* mutant studied in detail, RNA polymerase I and III subunits synthesized after a shift to the nonpermissive temperature could not be immunoprecipitated and were presumably degraded. It appears that RNA polymerases I and III assembled before the temperature shift sustained growth of mutant cells for several generations. Thus, the RPB3 and RPC5 subunits may play important roles in RNA polymerase assembly.

The amino terminus of the RPB3 subunit could be modified by the addition of amino acids that are recognized specifically by a monoclonal antibody. This alteration produced no detectable change in the growth properties of the cell. This epitope tag facilitated investigation of the effects of a mutation on the assembly of RNA polymerase II components by providing a highly specific and controlled assay for proteins associated with RPB3. This approach should also provide a rapid method to purify RNA polymerase II to homogeneity for future biochemical studies of the enzyme and its components.

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LITERATURE CITED

1. **Ahearn, J. M., Jr., M. S. Bartolomei, M. L. West, L. J. Cisek, and J. L. Corden.** 1987. Cloning and sequence analysis of the mouse genomic locus encoding the largest subunit of RNA polymerase II. J. Biol. Chem. **262**:10695–10705.
2. **Allison, L. A., M. Moyle, M. Shales, and C. J. Ingles.** 1985. Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell **42**:599–610.
3. **Barany, F.** 1985. Two-codon insertion mutagenesis of plasmid genes by using single-stranded hexameric oligonucleotides. Proc. Natl. Acad. Sci. USA **82**:4202–4206.
4. **Barnes, D., and J. Thorner.** 1986. Genetic manipulation of *Saccharomyces cerevisiae* by use of the *LYS2* gene. Mol. Cell. Biol. **6**:2828–2838.
5. **Biggs, J., L. L. Searles, and A. L. Greenleaf.** 1985. Structure of the eukaryotic transcription apparatus: features of the gene for the largest subunit of Drosophila RNA polymerase II. Cell **42**:611–621.
6. **Bitter, G. A.** 1983. Purification of DNA-dependent RNA polymerase II from *Saccharomyces cerevisiae*. Anal. Biochem. **128**:294–301.
7. **Boeke, J., F. LaCroute, and G. Fink.** 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197**:345–346.
8. **Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoro-orotic-acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154**:164–175.
9. **Carle, G., and M. Olson.** 1985. An electrophoretic karyotype for yeast. Proc. Natl. Acad. Sci. USA **82**:3756–3760.
10. **Carroll, S. B., and B. D. Stollar.** 1983. Conservation of a DNA-binding site in the largest subunit of eukaryotic RNA polymerase II. J. Mol. Biol. **170**:777–790.
11. **Chamberlin, M. J.** 1982. Bacterial DNA-dependent RNA polymerases, p. 61–86. *In* P. Boyer (ed.), The enzymes, vol. 15. Academic Press, Inc., New York.
12. **Chen, E., and P. Seeburg.** 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.
13. **Cho, J. M., and A. P. Kimball.** 1982. Probes of eukaryotic DNA-dependent RNA polymerase II. I. Binding of 9-beta-D-arabinofuranosyl-6-mercaptopurine to the elongation subsite. Biochem. Pharmacol. **31**:2575–2581.
14. **Dayhoff, M. O., W. C. Barker, and L. T. Hunt.** 1983. Establishing homologies in protein sequences. Methods Enzymol. **91**: 524–545.
15. **Elledge, S. J., and R. W. Davis.** 1988. A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. Gene **70**:303–312.
16. **Faust, D. M., R. Renkawitz-Pohl, D. Falkenburg, A. Gasch, S. Bialojan, R. Young, and E. Bautz.** 1986. Cloning and identification of the gene coding for the 140-kd subunit of *Drosophila* RNA polymerase II. EMBO J. **5**:741–746.
17. **Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler.** 1988. Purification of a *RAS*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. **8**:2159–2165.
18. **Frischauf, A.-M., H. Lehrach, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827–842.
19. **Grachev, M. A., G. R. Hartmann, T. G. Maximova, A. A. Mustaev, A. R. Schaffner, H. Sieber, and E. F. Zaychikov.** 1986. Highly selective affinity labelling of RNA polymerase B (II) from wheat germ. FEBS Lett. **200**:287–290.
20. **Ingolia, T. D., M. R. Slater, and E. A. Craig.** 1982. *Saccharomyces cerevisiae* contains a complex multigene family related to the major heat shock-inducible gene of *Drosophila*. Mol. Cell. Biol. **2**:1388–1398.
21. **Ishihama, A., N. Shimamoto, H. Aiba, K. Kawakami, H. Nashimoto, A. Tsugawa, and H. Uchida.** 1980. Temperature-sensitive mutations in the α subunit gene of *Escherichia coli* RNA polymerase. J. Mol. Biol. **137**:137–150.
22. **Kawakami, K., and A. Ishihama.** 1980. Defective assembly of ribonucleic acid polymerase subunits in a temperature-sensitive α-subunit mutant of *Escherichia coli*. Biochemistry **19**:3491–3495.
23. **Kunkel, T., J. Roberts, and R. Zakour.** 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
24. **Kuo, C.-L., and J. Campbell.** 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the *cdc8-1* mutation. Mol. Cell. Biol. **3**:1730–1737.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Lewis, M. K., and R. R. Burgess.** 1982. Eukaryotic RNA polymerases, p. 109–153. *In* P. Boyer (ed.), The enzymes, vol. 15. Academic Press, Inc., New York.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Mann, C., J.-M. Buhler, I. Treich, and A. Sentenac.** 1987. *RPC40*, a unique gene for a subunit shared between yeast RNA polymerases A and C. Cell **48**:627–637.
29. **Meinkoth, J., and G. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138**:267–284.

30. **Meiton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
31. **Memet, S., M. Gouy, C. Marck, A. Sentenac, and J.-M. Buhler.** 1988. *RPA190*, the gene coding for the largest subunit of yeast RNA polymerase A. J. Biol. Chem. **263**:2830–2839.
32. **Ng, R., and J. Abelson.** 1980. Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77**:3912–3916.
33. **Nonet, M., C. Scafe, J. Sexton, and R. A. Young.** 1987. Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol. Cell. Biol. **7**:1602–1611.
34. **Ovchinikov, Y. A., V. M. Lipkin, N. N. Modyanov, O. Y. Chertov, and Y. V. Smirnov.** 1977. Primary structure of alpha-subunit of DNA-dependent RNA polymerase from Escherichia coli. FEBS Lett. **76**:108–111.
35. **Paule, M. R.** 1981. Comparative subunit composition of the eukaryotic nuclear RNA polymerases. Trends Biochem. Sci. **6**:128–131.
36. **Sentenac, A.** 1985. Eukaryotic RNA polymerases. Crit. Rev. Biochem. **18**:31–91.
37. **Struhl, K., and D. E. Hill.** 1987. Two related regulatory sequences are required for maximal induction of *Saccharomyces cerevisiae HIS3* transcription. Mol. Cell. Biol. **7**:104–110.
38. **Sweetser, D., M. Nonet, and R. A. Young.** 1987. Prokaryotic and eukaryotic RNA polymerases have homologous core subunits. Proc. Natl. Acad. Sci. USA **84**:1192–1196.
39. **Vilamitjana, J., T. Baltz, D. Baltz, and C. Barreau.** 1983. Probing eukaryotic RNA polymerases B with monoclonal antibodies. FEBS Lett. **158**:343–348.
40. **Woychik, N., and R. A. Young.** 1989. RNA polymerase II subunit RPB4 is essential for high- and low-temperature yeast cell growth. Mol. Cell. Biol. **9**:2854–2859.
41. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
42. **Young, R. A., and R. W. Davis.** 1983. Yeast RNA polymerase II genes: isolation with antibody probes. Science **222**:778–782.
43. **Yura, T., and A. Ishihama.** 1979. Genetics of bacterial RNA polymerases. Annu. Rev. Genet. **13**:59–97.