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RNA Polymerase II Subunit RPB4 Is Essential for High- and Low-Temperature Yeast Cell Growth

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***RPB4* encodes the fourth-largest RNA polymerase II subunit in *Saccharomyces cerevisiae*. The *RPB4* gene was cloned and sequenced, and its identity was confirmed by amino acid sequence analysis of tryptic peptides from the purified subunit. The *RPB4* DNA sequence predicted a protein of 221 amino acids with a molecular mass of 25,414 daltons. The central 100 amino acids of the RPB4 protein were found to be similar to a segment of the major sigma subunit in *Escherichia coli* RNA polymerase. Deletion of *RPB4* produced cells that were heat and cold sensitive but could grow, albeit slowly, at intermediate temperatures. RNA polymerase II lacking the RPB4 subunit exhibited markedly reduced activity in crude extracts in vitro. The RPB4 subunit, although not essential for mRNA synthesis or enzyme assembly, was essential for normal levels of RNA polymerase II activity and indispensable for cell viability over a wide temperature range.**

RNA polymerase II is a complex enzyme whose multisubunit architecture is highly conserved among eucaryotes (16, 20). The enzyme consists of two large subunits and six to nine small subunits. *Saccharomyces cerevisiae* RNA polymerase II is among the best studied of the eucaryotic polymerases. The enzyme is composed of 10 polypeptides, encoded by *RPB1-10*, whose apparent molecular sizes are 220, 150, 44.5, 32, 27, 23, 16, 14.5, 12.6, and 10 kilodaltons (kDa). The 27-, 23-, 14.5-, and 10-kDa polypeptides are shared by all three nuclear RNA polymerases; the remaining polypeptides are unique to RNA polymerase II.

Important clues to RNA polymerase II subunit functions have come from a combination of functional analysis of the eucaryotic enzyme and the discovery that the *RPB1*, *RPB2*, and *RPB3* subunits are structural homologs of the *Escherichia coli* RNA polymerase core subunits β′, β, and α, respectively (2, 23; P. A. Kolodziej and R. A. Young, submitted for publication). *RPB1* and β′ probably play major roles in DNA binding (5, 6, 30). *RPB2* and β are largely responsible for binding nucleoside triphosphates (6, 7, 30). The β and β′ subunits share the catalytic site for RNA synthesis (6, 30), which suggests that *RPB1* and *RPB2* also share this function. The *RPB3* and α subunits have both been implicated in RNA polymerase assembly (11, 12; Kolodziej and Young, submitted).

The functions of the seven smaller subunits of RNA polymerase II are unknown. It is not clear whether all of these polypeptides are functional subunits, since some of the smaller polypeptides may be degradation products or proteins that simply copurify with RNA polymerase II. However, the conservation of the small proteins among eucaryotic RNA polymerase II enzymes (20) and the fact that antibodies directed against many of the small RNA polymerase II subunits can inhibit transcription in vitro (4, 25, 26) suggest that these proteins do have a role in the mRNA transcription apparatus.

We have isolated, characterized, and investigated the consequences of deletion of the yeast gene encoding the fourth-largest RNA polymerase II subunit, *RPB4*. Our results indicate that the RPB4 subunit is not essential for enzyme assembly or mRNA catalysis but is required for wild-type enzyme activity.

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MATERIALS AND METHODS

Yeast strains and media. *S. cerevisiae* N113 (DBY1826) (*MATa ade2 his3Δ200 leu2-112 ura3-52*) and N114 (DBY1827) (*MATa his3Δ200 leu2-3 leu2-112 ura3-52*) were from D. Botstein (Massachusetts Institute of Technology Cambridge, Mass.). The strains were grown on YPD medium (2% yeast extract, 1% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 2% glucose); YPD plates contained 2% agar. Dropout medium minus histidine (used to select for the *RPB4* disruption) has been described elsewhere (15). The yeast strain X2180-2 is a diploid derivative of the wild-type haploid strain S288C (*MATα mal gal2*).

Construction of the *RPB4* deletion mutant. *RPB4Δ1::HIS3* was constructed as described by Rothstein (18). A 2.4-kilobase-pair (kbp) *Bam*HI fragment containing the *RPB4* gene (Fig. 1) was inserted into the *Bam*HI site of YIp5 (3) to produce plasmid pRP41. pRP41 was cut with the restriction endonuclease *Spe*I, and the ends were filled in by using nucleoside triphosphates and reverse transcriptase. After partial digestion with *Eco*RI, a 7.3-kbp fragment was purified and ligated to the *HIS3*-containing DNA fragment with an *Eco*RI site at one end and a blunt end at the other; the resulting plasmid, pRP42, was recovered from transformed *E. coli* cells. The diploid N113 × N114 was then transformed with the *Bam*HI DNA fragment containing the *HIS3* substitution of *RPB4*. His^+ transformants were sporulated, and tetrad analysis was performed as previously described (21). Genomic DNA was prepared (21) and subjected to Southern analysis to verify the substitution of the chromosomal copy of *RPB4* with *HIS3*.

Recombinant DNA libraries. Construction of the yeast genomic DNA library in λgt11 has been described (22, 29). The λEMBL3A yeast genomic DNA library was a gift of M. Snyder (Stanford University School of Medicine, Stanford, Calif.). Both DNA libraries were constructed by using DNA from *S. cerevisiae* S288C. Libraries were probed with antibodies and radiolabeled DNA as described by Young et al. (27) and Davis et al. (8), respectively.

DNA sequence analysis. The 2.4-kbp DNA fragment from

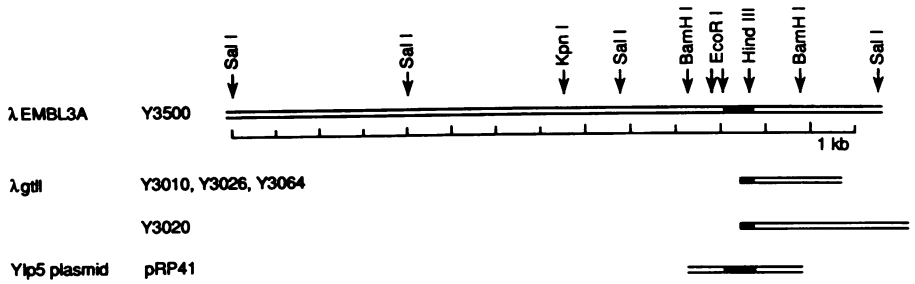

FIG. 1. Organization of *RPB4* DNA. The restriction maps of insert DNAs of various λgt11 and plasmid clones are aligned with that determined for the λEMBL3A clone Y3500. ■, RPB4 protein-coding sequences.

pBP41 was cloned into the vector M13mp18 or M13mp19. Overlapping subclones were constructed by using the Cyclone Biosystem (International Biotechnologies, Inc., New Haven, Conn.) and sequenced by using dideoxynucleotide chain termination reactions, followed by gel electrophoresis (19). DNA sequences were determined for both strands of DNA. Computer analysis of the sequence was performed by using the FASTA program (17) and IALIGN, an interactive version of the National Biomedical Research Foundation ALIGN program (9). Using IALIGN, the alignment score was obtained from 1,000 random runs of the RPB4 and σ^{70} amino acid sequences shown in Fig. 3, using 5 for the matrix bias and break penalty values.

Protein sequence analysis. Yeast RNA polymerase II was purified from *S. cerevisiae* X2180-2 by using protocols described by Valenzuela et al. (24). Amino acid sequence was obtained for purified *S. cerevisiae* RNA polymerase II by the method of Aebersold et al. (1). The purified enzyme was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose as described by Matsudaira (13), and stained with Ponceau S to visualize individual subunit bands. Nitrocellulose containing the blotted RPB4 protein was destained and digested with trypsin, and tryptic peptides were purified by high-performance liquid chromatography. Amino acid sequence was obtained from chromatography column fractions containing individual RPB4 peptides.

In vitro transcription assays. Cell extracts were prepared from 50-ml samples of cells grown to an optical density at 600 nm of 0.4 to 1.0. Cells were pelleted, rinsed with 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, and suspended in 1 ml of cold extraction buffer (0.05 M Tris hydrochloride [pH 8.0], 0.01 M $MgCl_2$, 0.5 mM EDTA, 0.01 M 2-mercaptoethanol, 0.3 M ammonium sulfate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1% dimethyl sulfoxide) plus 0.5 volume of acid-washed glass beads and transferred to glass tubes. Lysis of the cells was achieved by 10 vortex pulses, each lasting 10 s, followed by a 1-min incubation on ice. After centrifugation to remove cell debris, the protein concentration was measured and transcription assays were performed as described by Nonet et al. (14), using polycytidylic acid as a template.

RESULTS

Isolation of *RPB4*. Candidate RNA polymerase subunit genes were isolated by probing a λgt11 *S. cerevisiae* DNA library with polyclonal antiserum directed against purified yeast RNA polymerase II. Four clones produced signals when reprobed with antibodies directed against the 32-kDa RNA polymerase II polypeptide. The clones were mapped with restriction endonucleases and found to overlap (Fig. 1). Since the λgt11 clones produced an antibody-reactive β-galactosidase fusion protein, they probably lacked sequences for the amino-terminal portion of *RPB4*. To obtain DNA for the entire *RPB4* gene, insert DNA from clone Y3010 was used to screen an *S. cerevisiae* λEMBL3A genomic DNA library, from which one clone (Y3500) was selected for restriction mapping and DNA sequence analysis (Fig. 1). Alignment of the λgt11 and λEMBL3A insert DNAs allowed us to deduce the location of the candidate *RPB4* gene. The 2.4-kbp *Bam*HI restriction fragment from Y3500 was subcloned and sequenced. The DNA sequence contained an open reading frame for a 221-amino-acid protein with a molecular mass of 25,414 Da (Fig. 2).

To verify that the sequenced DNA encodes the RPB4 subunit, we obtained amino acid sequence from tryptic peptides generated from the purified 32-kDa subunit. Amino acid sequence analysis of one peptide produced the sequence ELSNLETLY. This sequence is identical to that

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TCAGAAGGGCGGTTAGGGTTAATAAAGCCATCGCGCTATTTTGATATTTTTATTAGGCCT   60

TTTATATTTTTTTTAGTTTTTTCTCCAAATCCTAAATCCTTTTTAGTGTCATTTACGTG   120

CAACGCGACAATAGTGATATTGTCATTCTTCAAACTTTTTTGCAAGGTAGCTATTAAATA   180

TACCTACACACATACGTACATGCTTAACGGTGAGACAAAAGTAAAGAAAGAGACTTGAAT   240

GGGTTGCTGCATCTTAATAAGTTGAGTTTGAGGGAGAAAGTACACAAATATTCATCCGTA   300

                                  M  N  V  S  T  S  T  F  Q  T
TTGAAAATATATATATAAGAATATAGAAAAATGAATGTTTCTACATCAACCTTTCAAACA   360

R  R  R  R  L  K  K  V  E  E  E  E  N  A  A  T  L  Q  L  G
AGACGGAGAAGATTGAAGAAAGTGGAGGAAGAAGAAAATGCAGCTACTCTACAACTGGGC   420

Q  E  F  Q  L  K  Q  I  N  H  Q  G  E  E  E  E  L  I  A  L
CAGGAATTCCAGCTGAAACAGATAAATCATCAGGGTGAAGAGGAGGAATTGATTGCCTTG   480

N  L  S  E  A  R  L  V  I  K  E  A  L  V  E  R  R  R  A  F
AACCTAAGTGAAGCCAGGTTAGTAATCAAAGAAGCTCTTGTAGAACGTAGGAGAGCATTT   540

K  R  S  Q  K  K  H  K  K  K  H  L  K  H  E  N  A  N  D  E
AAAAGATCGCAAAAAAAACACAAGAAGAAGCATTTGAAGCACGAAAACGCCAATGATGAA   600

T  T  A  V  E  D  E  D  D  D  L  D  E  D  D  V  N  A  D  D
ACTACGGCAGTAGAGGATGAAGATGATGATCTGGATGAAGATGACGTCAACGCTGATGAT   660

D  D  F  M  H  S  E  T  R  E  K  E  L  E  S  I  D  V  L  L
GATGATTTTATGCATTCTGAAACTAGGGAGAAGGAGTTGGAGTCTATCGACGTTCTGTTA   720

E  Q  T  T  G  G  N  N  K  D  L  K  N  T  M  Q  Y  L  T  N
GAACAGACAACGGGAGGAAATAATAAAGATTTGAAAAATACCATGCAGTATTTAACAAAT   780

F  S  R  F  R  D  Q  E  T  V  G  A  V  I  Q  L  L  K  S  T
TTCTCCCGATTTAGAGACCAAGAAACCGTCGGGGCAGTTATACAGCTTCTGAAAAGCACT   840

G  L  H  P  F  E  V  A  Q  L  G  S  L  A  C  D  T  A  D  E
GGGTTACATCCTTTTGAAGTGGCGCAACTAGGTTCTTTGGCCTGTGACACAGCTGATGAA   900

A  K  T  L  I  P  S  L  N  N  K  I  S  D  D  E  L  E  R  I
GCAAAGACTTTAATTCCAAGCTTAAACAATAAAATATCAGACGATGAGTTGGAAAGGATA   960

L  K  E  L  S  N  L  E  T  L  Y
CTAAAGGAATTGTCAAACCTAGAAACACTCTATTAATAACTGTATGTATACGTGTGTGTA   1020

ACTAGTAATGAATTTTCACCCTGTGGAAGTTAGGCCTATTTAACAGGAAAAAGGACAGGA   1080

TTTTAAATCTCGTCTTGCTCTTTTAAGAAATATTTGCCGCTACCGTTTTTCCCACCATCT   1140
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FIG. 2. Sequence of *RPB4* DNA and the predicted amino acid sequence of the RPB4 subunit. The amino acid sequence obtained from an RPB4 tryptic peptide is underlined.

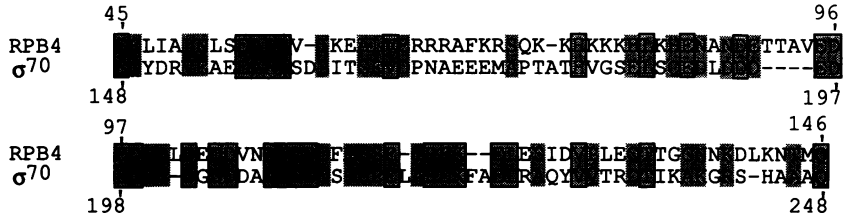

FIG. 3. Sequence similarities between portions of the RPB4 protein and the 70-kDa *E. coli* RNA polymerase σ subunit. Identical amino acid residues are boxed and shaded; conservative amino acid replacements (those that scored +1 or greater in the mutation data matrix of Dayoff et al. [9]) are shaded. The 70-kDa σ subunits from *Salmonella typhimurium* and *E. coli* RNA polymerases have identical amino acid sequences in the region represented.

predicted by the DNA sequence for the nine carboxy-terminal amino acids of RPB4 (Fig. 2).

The molecular mass of RPB4 predicted by its sequence (25.4 kDa) is considerably less than that estimated by SDS-polyacrylamide gel electrophoresis (32 kDa). The sequences of *RPB1*, *RPB2*, and *RPB3* also predict subunit molecular masses (190, 142, and 38 kDa) that are less than their apparent molecular masses estimated by SDS-polyacrylamide gel electrophoresis (220, 150, and 44.5 kDa, respectively). Phosphorylation or glycosylation of these proteins may account for some of these differences. However, the unusual concentration of first basic and then acidic amino acid residues in the middle of the protein might also influence the properties of the protein on SDS-polyacrylamide gels. Many bacterial σ factors are known to migrate anomalously on SDS-polyacrylamide gels (10).

Comparison of the amino acid sequence of RPB4 with others in the GenBank data base revealed that a segment of RPB4 was similar to a portion of the predominant σ subunit (σ^{70}) of *E. coli* and *Salmonella typhimurium* RNA polymerases (Fig. 3). A 102-amino-acid stretch of the RPB4 protein was found to be similar to a 101-residue segment of the bacterial σ subunits. To assess the significance of this sequence similarity, the two sequences were subjected to computerized statistical analysis. Comparison of the alignment score for the RPB4 and σ^{70} sequences shown in Fig. 3 with 1,000 random permutations of the same amino acids revealed that the alignment score was 3.6 standard deviations from the mean.

***RPB4* is a single-copy gene.** The copy number of *RPB4* in the yeast haploid genome was investigated by probing whole genomic DNA by Southern analysis (Fig. 4). The 2.4-kbp *Bam*HI DNA fragment of plasmid pRP41 was labeled and used to probe immobilized yeast genomic DNA restriction fragments. The pattern of hybridization, which did not change over a range of hybridization and wash conditions, revealed that the *RPB4* gene was present only once in the yeast genome. The *RPB4* gene was localized to chromosome X by probing a Southern blot containing *S. cerevisiae* chromosomes separated by pulsed-field electrophoresis (not shown).

***RPB4* deletion causes conditional lethality.** The genomic copy of *RPB4* was replaced with a selectable marker to determine whether *RPB4* is essential for cell viability (Fig. 5A). All but 93 bp of the *RPB4* coding sequence in plasmid pRP41 was replaced with *HIS3*. Diploid cells were transformed with the *Bam*HI fragment containing the *HIS3* replacement of *RPB4*, and His^+ cells were isolated, sporulated, and subjected to tetrad analysis. Spores containing *RPB4Δ1*::*HIS3* germinated but grew slower than *RPB4* wild-type cells at 24°C (Fig. 5B). DNA prepared from the diploid parent and from the His^+ and His^- haploid progeny was subjected to Southern analysis (Fig. 5C). The analysis confirmed that DNA from His^+ cells contained *RPB4Δ1*::*HIS4* and that His^- cells contained an intact *RPB4* gene. Western analysis of extracts prepared from wild-type and *RPB4Δ1*::*HIS3* cells demonstrated that cells lacking the *RPB4* gene also lacked the RPB4 protein (not shown). Thus, *RPB4* was not essential for cell viability at 24°C, but its absence affected the cell growth rate.

RPB4Δ1::*HIS3* cells were plated at various temperatures

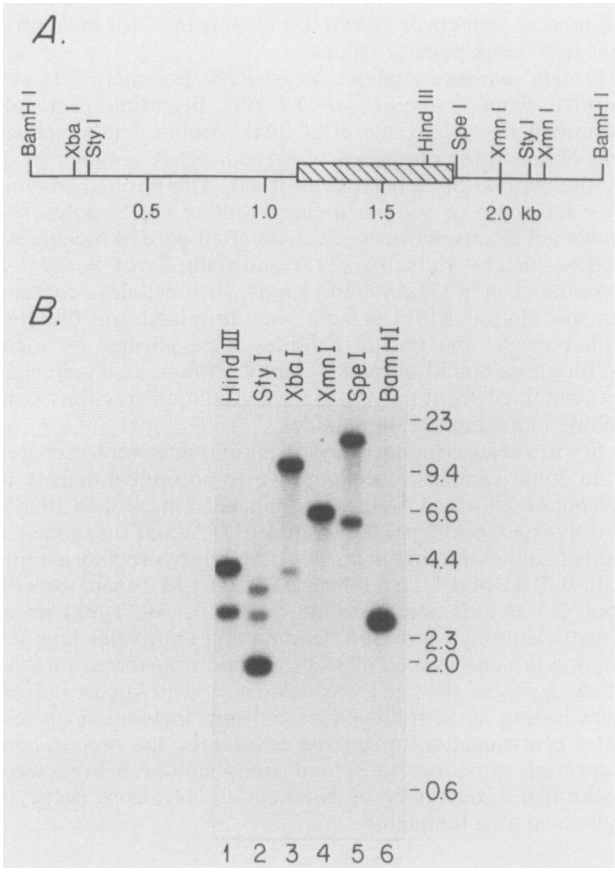

FIG. 4. *RPB4* copy number. (A) Detailed restriction map of the 2.4-kbp *Bam*HI fragment containing *RPB4* sequences (Fig. 1), which was used as a radiolabeled probe. ▨, *RPB4* coding region. (B) Southern analysis of yeast DNA from wild-type haploid cells digested with various restriction enzymes and probed with the 2.4-kbp *Bam*HI fragment. The filter was washed four times in 2× SSPE–0.2% SDS (8) at 45°C; washes at higher and lower stringency produced the same result. Sizes of the DNA markers (shown on the right) are expressed in kilobase pairs.

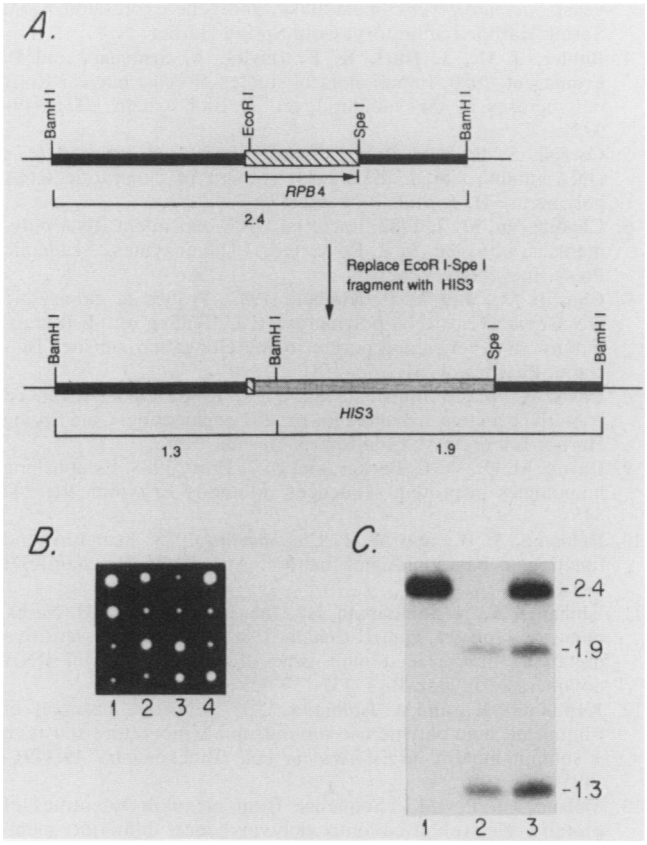

FIG. 5. Construction and genetic analysis of an *RPB4* deletion. (A) Replacement of *RPB4* with the *HIS3* gene. (B) Dissected tetrads from a diploid cell containing one copy of *RPB4* and one copy of *RPB4Δ1::HIS3*. (C) Southern analysis of *Bam*HI-digested whole genomic DNA prepared from a His^- haploid colony (*RPB4*) (lane 1), a His^+ haploid colony (*RPB4Δ1::HIS3*) (lane 2), and the parental diploid (*RPB4/RPB4Δ1::HIS3*) (lane 3). The probe was the 2.4-kbp *Bam*HI DNA fragment shown in panel A. DNA sizes (shown on the right) are expressed in kilobase pairs.

to investigate their temperature ranges for growth. Similar numbers of wild-type and *RPB4Δ1::HIS3* cells were spotted on YPD plates, and the plates were incubated at 12, 24, 30, 32, and 36°C (Fig. 6). The results revealed that *RPB4* was not essential for growth at 24 and 30°C but was essential at lower (12°C) and higher (32 and 36°C) temperatures.

Cells lacking *RPB4* exhibit a variety of defects even at permissive temperatures. In addition to having slow growth rates, they have a long lag phase and reach stationary phase at lower cell densities than do wild-type cells. The mutant cells are also unable to grow on plates deficient in the sugar alcohol inositol, which is utilized in the synthesis of phosphoglycerides. RNA polymerase II subunit mutations frequently produce inositol auxotrophy; most *RPB1* and *RPB2* temperature-sensitive mutants require inositol for growth (C. Scafe, M. Nonet, and R. A. Young, unpublished data).

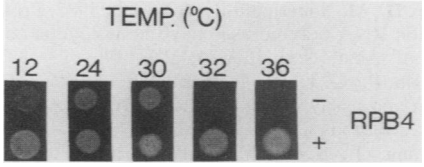

FIG. 6. Effect of temperature on growth of *RPB4Δ1::HIS3* cells. Equal numbers of *RPB4Δ1::HIS3* (−RPB4) and wild-type (+RPB4) cells were spotted onto YPD medium and incubated at the indicated temperatures.

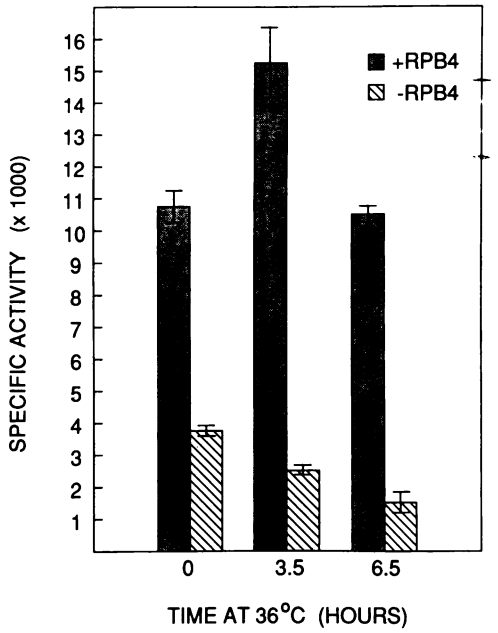

FIG. 7. RNA polymerase II activity in crude extracts in vitro. Extracts were prepared from cells grown at 24°C (0 h) and from cells shifted to 36°C for 3.5 or 6.5 h. Specific activity is defined as counts per minute incorporated into trichloroacetic acid-precipitable material per 50 μg of total protein in a 15-min assay performed at 24°C.

To eliminate the possibility that the presence of 93 bp of *RPB4* in *RPB4Δ1::HIS3* contributed to the growth phenotypes of the deletion mutant, the entire coding region and 0.6 kbp of upstream sequences were replaced with *HIS3* to produce the null allele, *RPB4Δ2::HIS3*. Haploid cells with this allele exhibited the same phenotypes as did cells with *RPB4Δ1::HIS3*, indicating that both mutations produced null alleles.

RNA polymerase II lacking *RPB4* has reduced activity in vitro. RNA polymerase II elongation activity was measured in crude extracts of wild-type and *RPB4Δ1::HIS3* cells (Fig. 7). Cells were grown at 24°C to logarithmic phase and harvested or shifted to 36°C for 3.5 or 6.5 h before harvesting. Crude extracts were prepared, and equivalent amounts of protein were added to template mixtures containing [^{3}H]GTP. The reaction was allowed to proceed for 15 min at 24°C. The absence of RPB4 protein had a marked effect on the elongation activity of RNA polymerase II. [^{3}H]GTP incorporation by mutant extracts was 30% of the wild-type value. RNA polymerase II activity in mutant extracts made from cells shifted to 36°C and assayed at 24°C was only 13 to 16% that in wild-type extracts (Fig. 7). In contrast, the levels of RNA polymerase I and III activities were similar in extracts of *RPB4* mutant and wild-type cells (not shown).

DISCUSSION

We have cloned and characterized the gene encoding the fourth-largest subunit of yeast RNA polymerase II, *RPB4*. *RPB4* encodes a 25.4-kDa subunit that is not essential for

yeast cell growth. Cells lacking *RPB4* grow slowly at permissive temperatures (24 to 30°C) and cease growth at high (32°C) and low (12°C) temperatures. RNA polymerase II activity in extracts from *RPB4*-deficient cells is lower than in wild-type extracts.

A portion of the amino acid sequence of the RPB4 subunit is similar to a segment of a component of the procaryotic transcription apparatus. The 102 residues in the middle of the 221-amino-acid protein are similar to a 101-residue segment of the 70-kDa σ subunits of *E. coli* and *Salmonella typhimurium* RNA polymerases (Fig. 3). The function of this segment of σ is not yet defined. The 101-amino-acid region is not present in minor σ subunits (10), but these minor σ factors may not possess all of the functions of the major σ subunit.

An unusual feature of the first half of the RPB4 subunit is the presence of strings of residues with basic side chains alternating with strings of residues with acidic side chains. For example, more than half of amino acids 56 through 84 are lysine, arginine, or histidine residues, while more than half of amino acids 85 through 112 are aspartate and glutamate (Fig. 3). These adjacent basic and acidic domains may interact with one another or with other components of the mRNA transcription apparatus.

The ability of cells lacking *RPB4* to grow, even if slowly and in a narrow temperature range, indicates that the RPB4 subunit is unlikely to be involved directly in the initiation of mRNA synthesis, mRNA catalysis, or the assembly of RNA polymerase II. The slow and temperature-sensitive growth phenotypes of *RPB4*-deficient cells suggests that RNA polymerase II requires the RPB4 subunit for maximal efficiency and thermostability. The observation that mutant RNA polymerase II activity is lower in vitro than wild-type activity is consistent with this idea.

Genes for the four largest of the yeast RNA polymerase II subunits have now been isolated and characterized (2, 23, 28; Kolodziej and Young, submitted). Each of these genes exists in single copy in the haploid yeast genome, and they reside on different chromosomes. *RPB1*, *RPB2*, and *RPB3* are indispensable for mRNA synthesis in vivo, and *RPB4* is essential for normal levels of RNA polymerase II activity and cell growth over a broad temperature range.

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