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General requirement for RNA polymerase II holoenzymes *in vivo*

(transcription initiation/conditional mutants)

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ABSTRACT **Yeast RNA polymerase II holoenzymes have been described that consist of RNA polymerase II, a subset of general transcription factors, and nine SRB regulatory proteins. The feature that distinguishes the RNA polymerase II holoenzymes from other forms of RNA polymerase II in the cell is their tight association with SRB proteins. We investigated the fraction of genes that require SRB proteins *in vivo* by examining the effect of temperature-sensitive mutations in *SRB* genes on transcription by RNA polymerase II. Upon transfer to the restrictive temperature, there is a rapid and general shutdown of mRNA synthesis in *srb* mutant cells. These data, combined with the observation that essentially all of the SRB protein in cells is tightly associated with RNA polymerase II molecules, argue that SRB-containing holoenzymes are the form of RNA polymerase II recruited to most promoters in the cell.**

Selective transcription initiation by RNA polymerase II requires the action of at least five general transcription factors: TATA-binding protein, TFIIB, TFIIE, TFIIF, and TFIIH (1–3). These factors and RNA polymerase II can assemble in an ordered fashion onto promoter DNA *in vitro* (2–6), suggesting how transcription initiation may occur *in vivo*. Genetic and biochemical studies in yeast have recently challenged this paradigm, however, by identifying a large preassembled complex, an RNA polymerase II holoenzyme, that may be the physiologically relevant form of the transcription initiation apparatus (7–9). These RNA polymerase II holoenzymes contain RNA polymerase II, a subset of general transcription factors, and nine SRB proteins (8, 41–43). The general transcription factors found associated with the largest of these holoenzymes (8) were previously found to stably interact with RNA polymerase II in the absence of DNA (2, 10–18). The RNA polymerase II holoenzymes are capable of site-specific initiation when supplemented with the general factors not tightly associated with the purified holoenzyme. They are also responsive to activators (8, 9), a feature not observed with purified RNA polymerase II and general transcription factors alone (19, 20).

We have proposed (8, 43) that the RNA polymerase II holoenzyme is a form of the enzyme that is readily recruited to promoters *in vivo*. However, because only a portion of RNA polymerase II in cells is found in holoenzyme form (8, 9), it is unclear whether all RNA polymerase II genes require the holoenzyme for transcription initiation.

Conditional temperature-sensitive (ts) mutations in the transcription initiation apparatus have been used (21–25) to investigate the influence of specific factors on mRNA synthesis *in vivo* and to study the fraction of promoters that require these factors (21–25). This approach, in which transcript levels are monitored in cells undergoing a shift to the restrictive temperature, was used to demonstrate that the *Escherichia coli* σ^{70}-containing RNA polymerase holoenzyme is the form of RNA polymerase responsible for the synthesis of most cellular transcripts *in vivo* (21). We reasoned that because *Saccharomyces cerevisiae* holoenzymes contain essentially all of the SRB protein in the cell (7, 8, 41–43), the fraction of genes that employ RNA polymerase II holoenzymes *in vivo* could be investigated by studying the effects of ts mutations in *SRB* genes on transcription of mRNA. At the restrictive temperature, *srb* mutant cells rapidly cease mRNA synthesis. These findings indicate a general requirement for SRBs and the RNA polymerase II holoenzyme in transcription.

MATERIALS AND METHODS

Yeast Strains and Plasmids. The recessive *srb4-138* mutant allele was generated by using a PCR-based mutagenesis strategy (26) and isolated by using plasmid shuffle techniques (27). Isogenic wild-type (Z579) and *srb4-138* (Z628) strains contain complete deletions of the *SRB4* coding region (7) covered by the plasmids pCT127 (*SRB4 LEU2 CEN*) and pCT181 (*srb4-138 LEU2 CEN*), respectively. The *rpb1-1* strain (Z676) contains a deletion of the *RPB1* coding region covered by the plasmid RY2522 (*rpb1-1 URA3 CEN*) (22).

RNA Analysis. Total RNA from cells was isolated by using hot phenol extraction (28). RNA was quantified by absorbance at 260 nm and the integrity of the RNA confirmed by ethidium bromide staining of RNA in agarose gels. Slot blot analysis was performed as described (29). S1 nuclease protection assays were carried out with 5–30 μg of RNA and DED1, HIS3, TRP3, rRNA, and $tRNA^{w}$ oligonucleotide probes as described (23). The sequences for the other oligonucleotide probes are as follows: ACT1, GGAAGAGTACAAGGACAAAACGGCTTGGATGGAAACGTAGAAGGCATTCCA; CDC7, GGGGCTACTCTCGAAGATCCCGTCATTATGTACAGCAGGTTGAGCATGCCT; MET19, GCCTTACCGGCACGCATCATGATGGGGACGCCCTCCCAACGCTCGACACTT; RAD23, GCAGTGGCTGCAGGAGCTGCAGAAGCATCGGTACTGGGGGATGCAATCCA; STE2, GTCGACGGGTTCAACTTCTCCCTCTTTGTAACTTGCATCAGCAAACGGATGACA; TCM1, GGAGTGTCAACAACGGTGACAGCTTCGACAACTTCACGCTTGTGGTGAGCT. Oligonucleotides are written in the 5′-to-3′ direction and contain six residues at their 3′ ends that are not complementary to the RNA, permitting distinction between bands due to appropriate RNA·DNA hybrids and undigested probe.

RESULTS

Conditional *srb4* Mutant Rapidly Ceases Growth. To investigate whether the *S. cerevisiae* holoenzyme is the form of RNA polymerase II responsible for the synthesis of most cellular transcripts *in vivo*, we generated a ts mutation, *srb4-138*, in the essential *SRB4* gene. Cells containing this recessive mutation

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Abbreviation: ts, temperature sensitive.

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grew normally at the permissive temperature of 30°C but failed to grow at the restrictive temperature of 37°C (Fig. 1*A*). Upon shifting a growing culture of *srb4-138* cells to the restrictive temperature, cell growth rapidly decreased, failing to double before growth ceased altogether (Fig. 1*B*).

Conditional *srb4* Mutant Rapidly Ceases mRNA Synthesis. The effect of the *srb4-138* mutation on mRNA synthesis was investigated by allowing wild-type and mutant cells to grow at the permissive temperature and then shifting the cultures to the restrictive temperature. Aliquots of cells were taken immediately before and at various times after the shift and total RNA was prepared. The amount of poly(A)$^+$ mRNA obtained for each sample was determined by applying equivalent amounts of RNA to a slot blot and probing with labeled poly(T) (Fig. 2). Results from this type of assay have been shown by others to accurately reflect the relative amounts of poly(A)$^+$ mRNA in yeast cells (29). The standardization of samples with respect to total RNA is adequate to permit comparisons of mRNA accumulation because the vast majority of the RNA in a typical eukaryotic cell is composed of highly stable rRNA (75%) and tRNA (15%), whereas less than 5% of the total RNA is composed of relatively unstable mRNA (30). After the shift to the restrictive temperature, poly(A)$^+$ mRNA levels declined dramatically and rapidly in mutant cells relative to wild type (Fig. 2). The half-life of the *S. cerevisiae* poly(A)$^+$ mRNA population was previously found to be between 15 and 23 min (31–34); temperature shift experiments with a ts RNA polymerase II mutant (*rpb1-1*) were among the approaches used to ascertain the average half-life of the yeast mRNA population (34). We estimate that the half-life of the mRNA population in *srb4-138* cells shifted to the restrictive temperature is between 15 and 20 min. These kinetics are consistent with a general shutdown in transcription by RNA polymerase II.

The defect in RNA polymerase II transcription was examined in more detail by investigating the effect of a temperature shift on synthesis of selected mRNAs (Fig. 3). Total RNA from wild-type and mutant cells was hybridized with an excess of labeled oligonucleotides complementary to specific transcripts, and the resulting products were treated with S1 nuclease and subjected to denaturing polyacrylamide gel electrophoresis. The nine messages selected for analysis, from genes *ACT1, CDC7, DED1, HIS3, MET19, RAD23, STE2, TCM1*, and *TRP3*, represent a broad spectrum of genes affecting diverse cellular processes. Since this approach measures steady-state levels of mRNAs, the absence of new mRNA synthesis would lead to reduced transcript levels, the rate of reduction reflecting the mRNA decay rate. In *srb4* mutant cells, the levels of all of the mRNAs declined after temperature shift at a rate that correlates well with decay rates observed by other investigators (22–25, 34, 35). In contrast, the data indicate that these transcripts continue to be synthesized in wild-type cells throughout the entire 4-h period at 37°C. These results are consistent with those obtained by analyzing total poly(A)$^+$ RNA and indicate a general shutdown in mRNA synthesis in *srb4* mutant cells at the restrictive temperature.

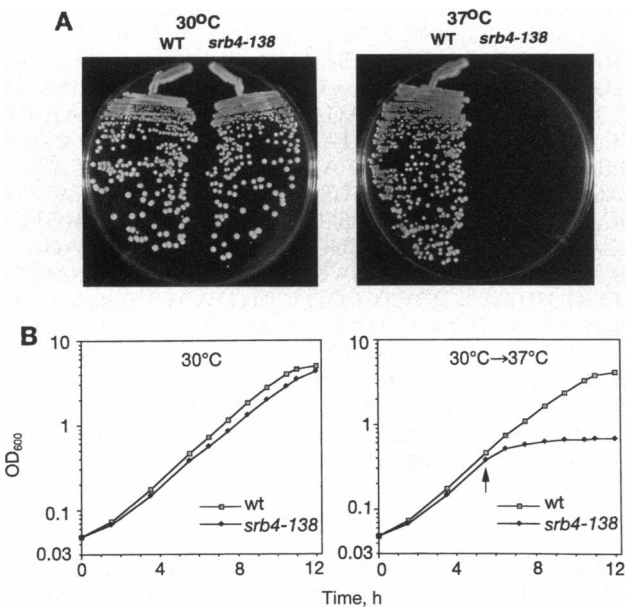

FIG. 1. Conditional *srb4* mutant rapidly ceases growth. (*A*) Growth of isogenic wild-type (Z579) and *srb4-138* (Z628) strains on YPD plates at 30°C (*Left*) and 37°C (*Right*). (*B*) Growth of wild-type and *srb4-138* strains in YPD medium as determined by measuring OD_{600} at various times. At the time indicated by the arrow, cultures growing at 30°C were divided, and half the culture was left at 30°C (*Left*) and the other half was shifted to 37°C (*Right*).

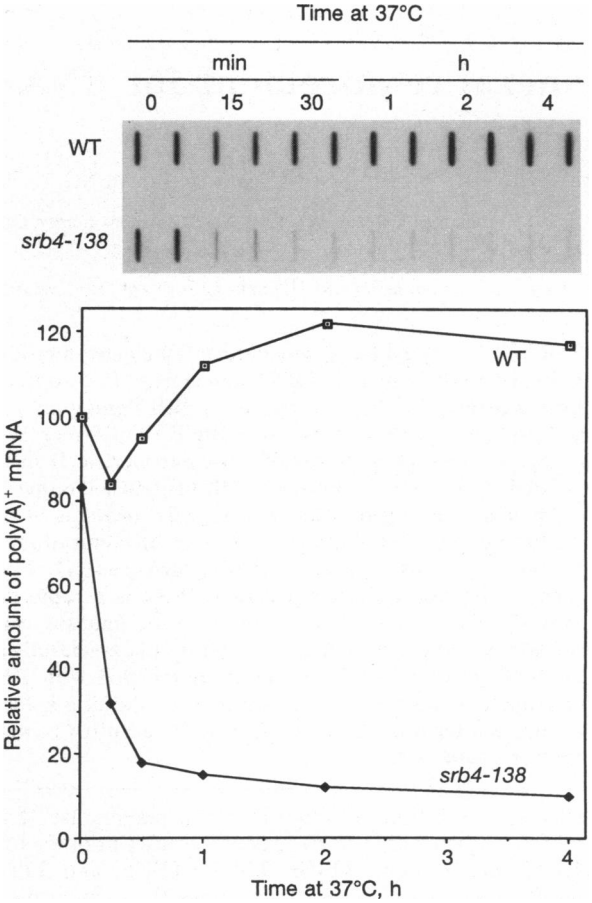

FIG. 2. mRNA levels rapidly decline in *srb4-138* cells at the restrictive temperature. At the times indicated, immediately before and after the shift from 30°C to 37°C, aliquots of cells were removed and total RNA was prepared. Equivalent amounts of RNA (2 μg) were applied to a slot blot, in duplicate, and the filter was probed with [^{32}P]poly(T) (*Upper*). The results were quantified by using a Fuji Bio-Image Analyzer and plotted (*Lower*). Each point represents the average value of the duplicate slots, normalized to a value of 100 for wild-type cells at time 0.

We carried out identical experiments with cells containing a ts mutation in the essential *SRB6* gene. Cells containing the ts mutation *srb6-107* also rapidly ceased general mRNA synthesis after a shift to the restrictive temperature (data not shown). It is important to note that both the *srb4* and *srb6* mutations used here produce recessive defects, indicating that the general shutdown in mRNA synthesis is not caused by defective holoenzymes blocking initiation by RNA polymerase II molecules lacking SRB proteins.

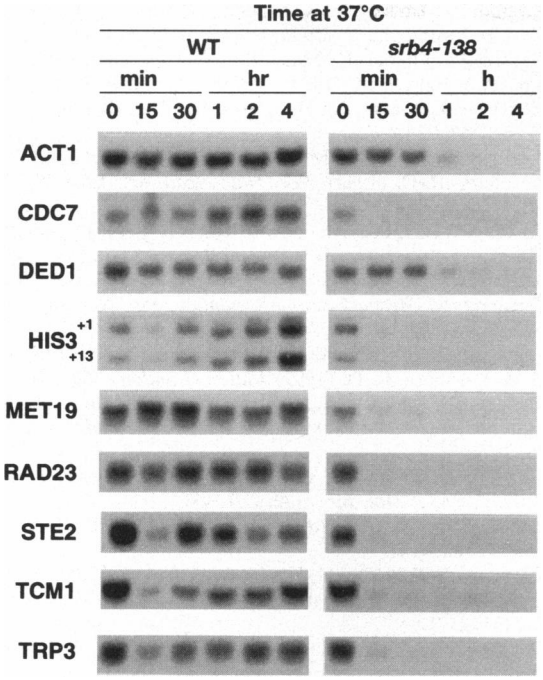

FIG. 3. Conditional *srb4* mutant rapidly ceases mRNA synthesis. At the times indicated, immediately before and after the shift from 30°C to 37°C, aliquots of cells were removed and total RNA was prepared. Equivalent amounts of RNA were hybridized with an excess of ^{32}P-labeled oligonucleotide complementary to the indicated transcripts, treated with S1 nuclease, and subjected to denaturing polyacrylamide gel electrophoresis. The *HIS3* oligonucleotide is complementary to the 5′ end of the message and detects transcripts initiated from the +1 and +13 sites. The half-lives of mRNAs from *ACT1, HIS3, STE2,* and *TCM1* genes have been determined to be 30, 7, 4, and 11 min, respectively (34). Very similar results were obtained for a subset of these mRNAs in an independent series of experiments (35). The precise half-lives of mRNAs from *CDC7, MET19, RAD23,* and *TRP3* genes have not been calculated but the decay of these messages in the absence of transcription has been investigated by others (22–25). The transient decrease in the levels of some of the transcripts from wild-type cells is due to a mild heat shock response (36).

Effect of Loss of SRB4 Activity on Transcription by RNA Polymerases I, II, and III. The SRBs were identified by the ability of mutations in these genes to specifically suppress conditional and auxotrophic phenotypes associated with truncations of the C-terminal domain of RNA polymerase II (7, 37, 38) and the vast majority of SRB protein in the cell is tightly associated with RNA polymerase II (7, 8). Nonetheless, we investigated the influence of the *srb4-138* mutation on rRNA synthesis by RNA polymerase I and tRNA synthesis by RNA polymerase III. tRNAs are extremely stable but their transcripts contain introns that are rapidly processed with half-lives of less than 3 min (23, 39). S1 nuclease analysis with an oligonucleotide complementary to the 5′-intron–exon junction of the tryptophan family of tRNA transcripts was used to measure RNA polymerase III activity (Fig. 4). There is no appreciable effect on the RNA polymerase III synthesis of tRNA by the *srb4-138* mutant.

The shutdown of RNA polymerase I-dependant rRNA synthesis under conditions when mRNA synthesis is affected is well-established and is thought to be a consequence of a stringent response (22–25). As expected, rRNA synthesis is significantly reduced in *srb4-138* mutant cells after the shift to the restrictive temperature (Fig. 4). rRNA synthesis was investigated by using S1 nuclease analysis with an oligonucleotide complimentary to the 3′ processing junction of the short-lived ribosomal precursor RNA (23, 40). This decrease in RNA polymerase I activity is similar to that observed in cells

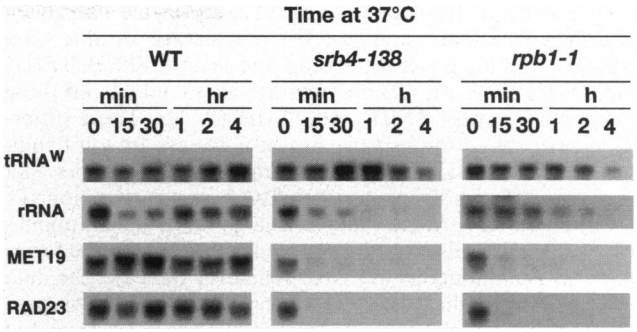

FIG. 4. Effect of loss of SRB4 activity on transcription by RNA polymerases I, II, and III. At the times indicated, immediately before and after the shift from 30°C to 37°C, aliquots of cells were removed and total RNA was prepared. Equivalent amounts of RNA were hybridized with an excess of ^{32}P-labeled oligonucleotide complementary to the indicated transcripts, treated with S1 nuclease, and subjected to denaturing polyacrylamide gel electrophoresis.

containing the ts *rpb1-1* allele of *RPB1*, the gene encoding the largest subunit of RNA polymerase II (Fig. 4) (22, 23). RNA polymerases II and III activities in *srb4-138* and *rpb1-1* cells are also nearly identical. In both of these mutants the synthesis of *MET19* and *RAD23* transcripts is dramatically reduced while the synthesis of tRNA is largely unaffected (Fig. 4).

DISCUSSION

The experiments described here demonstrate a general requirement for the SRB proteins in RNA polymerase II transcription *in vivo*. Cells containing ts mutations in the essential *SRB4* or *SRB6* genes rapidly cease mRNA synthesis at the restrictive temperature. Because the RNA polymerase II holoenzyme contains most of the SRB protein in the cell, we infer that the holoenzyme is the form of RNA polymerase II responsible for the majority of mRNA synthesis *in vivo*.

The rapid and general loss of mRNA in *srb* mutant cells at the restrictive temperature is likely due to the cessation of mRNA synthesis and not the result of a dramatic decrease in message stability. The half-life of the mRNA population and the kinetics of specific mRNA loss in *srb* mutant cells at the restrictive temperature are essentially identical to that observed in cells containing a ts mutation (*rpb1-1*) in RNA polymerase itself (22, 23, 34, 35). Cells containing this RNA polymerase II mutation are frequently used in experiments to measure mRNA half-life because they exhibit rapid transcriptional arrest (22) and because the rates determined with these cells are similar to those determined by other methods, such as approach to steady-state labeling or inhibition of transcription with drugs (34). The similarity in the kinetics of message decay in *srb4-138* and *rpb1-1* mutant cells at the restrictive temperature indicates that the reduction in mRNA levels is mostly, if not entirely, due to a cessation of mRNA synthesis.

The general cessation of mRNA synthesis in *srb* mutant cells is likely to be a direct result of loss of SRB activity. The remote possibility that the rapid cessation of mRNA synthesis was due to indirect effects, such as a shutdown in metabolic activity as cells stopped growing, or the loss of a highly unstable protein that is encoded by an unstable RNA whose synthesis is dependent on SRBs, was addressed by Cormack and Struhl (23). These investigators performed a similar set of temperature-shift experiments using a strain containing a ts mutation in *CDC28*, the gene encoding the cyclin-associated protein kinase that mediates entry into the cell cycle. The effects of cycloheximide, a potent inhibitor of cellular translation, on transcription of a subset of messages in wild-type cells were also examined. In both cases, no appreciable effects on RNA polymerase II transcription were observed.

Two forms of RNA polymerase II holoenzyme have been described, one that contains RNA polymerase II, nine SRB proteins, and the general transcription factors TFIIB, TFIIF, and TFIIH (7, 8, 41, 42) and another that contains all these components except TFIIB and TFIIH (9, 42). These differences may reflect differences in yeast strains, growth conditions, and conditions of protein fractionation, factors that appear to affect holoenzyme stability during purification (D. Chao, D. Jeffery, A. Koleske, J. Zhang, and R.A.Y., unpublished data). It is also possible that multiple holoenzyme forms may exist simultaneously *in vivo*. Whatever the case, the data presented here indicate that SRB-containing RNA polymerase holoenzymes are the form of RNA polymerase II utilized at most promoters *in vivo*. This conclusion is based upon the demonstration that SRB4 and SRB6 play a general role in RNA polymerase II transcription *in vivo* and the observation that the vast majority of the SRBs in the cell are tightly associated with RNA polymerase II holoenzymes (7, 8, 41–43). Although a small fraction of SRB protein must be in the process of assembly *in vivo*, essentially all of the SRB protein in cells copurifies with the holoenzyme molecules (7, 8, 41–43).

The results described here have additional implications for the mechanisms involved in regulation of transcription initiation. We previously estimated the levels of various SRB, RNA polymerase II, and general factor subunits in cells by using quantitative Western blot analysis on cell extracts. The SRB proteins accumulate to approximately 10% the levels of total cellular RNA polymerase II and TFIIB (8). Thus, the assembly of an RNA polymerase II holoenzyme is limited by the levels of SRB proteins. The SRBs may thus play a key regulatory role in holoenzyme formation prior to initiation complex assembly.

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