A Functional Role for the Switch 2 Region of Yeast RNA Polymerase II in Transcription Start Site Utilization and Abortive Initiation*

RNA polymerase II (RNAPII) is responsible for the synthesis of mRNA from eukaryotic protein-encoding genes. In this study, site-directed mutagenesis was employed to probe the function of residues within the *Saccharomyces cerevisiae* RNAPII active center in the mechanism of transcription start site utilization. We report here the identification of two mutations in the switch 2 region, *rpb1-K332A* and *rpb1-R344A*, which conferred conditional growth properties and downstream shifts in start site utilization. Analyses of double mutant strains demonstrated functional interactions between these switch 2 mutations and a mutation in the largest subunit of transcription factor IIF (TFIIF) that confers upstream shifts in start site usage. Importantly, biochemical analyses demonstrated that purified Rpb1-R344A mutant polymerase exhibited impaired ability to stabilize a short RNA-DNA hybrid in the active center, an increased frequency of abortive transcription in runoff assays, and both a downstream shift and increased abortive initiation in reconstituted transcription assays. These results provide evidence for a role of switch 2 during start site utilization and indicate that RNA-DNA hybrid stability at the 3’-end of the transcript is a determinant in this process. We discuss these results within the context of a proposed model regarding the concerted roles of RNAPII, TFIIB, and TFIIF during mRNA 5’-end formation in *S. cerevisiae*.

The synthesis of mRNA from protein-encoding (class II) genes in eukaryotic organisms is a regulated multistep process that involves the concerted action of RNA polymerase II (RNAPII) and a host of auxiliary transcription factors (1, 2). In higher eukaryotes, transcription initiation typically occurs ∼30 base pairs downstream of a TATA element. In contrast, mRNA 5’-ends in the yeast *Saccharomyces cerevisiae* frequently map to multiple sites within a window ranging from 40 to 120 base pairs or greater downstream of a TATA element (3, 4). Although the precise mechanism by which RNAPII recognizes and productively utilizes a potential start site remains to be elucidated, previous studies have firmly established that RNAPII and the general transcription factors IIB (TFIIB) and IIF (TFIIF) play important roles in determining the position of mRNA 5’-ends in *S. cerevisiae*. Numerous genetic and biochemical studies of *S. cerevisiae* TFIIB conducted in our laboratory and by others have shown that amino acid substitutions in a highly conserved N-terminal region of TFIIB can alter the position of mRNA 5’-ends both *in vivo* and *in vitro* (5–10). Specifically, these TFIIB mutations confer a downstream shift in start site utilization, i.e. diminished utilization of sites located proximal to the TATA element with accompanying enhanced utilization of sites located further downstream. Interestingly, the structural determination of an *S. cerevisiae* TFIIB-RNAPII co-crystal has revealed that this N-terminal region of TFIIB adopts a finger-like structure that projects into the RNA exit channel of the polymerase and is positioned to potentially make contact with a short RNA-DNA hybrid in the active center (11).

In contrast to the downstream shifts conferred by TFIIB mutations, we recently reported that mutations in either the Tfg1 or Tfg2 subunits of *S. cerevisiae* TFIIF can cause upstream shifts in the position of mRNA 5’-ends both *in vivo* and *in vitro* (12). Upstream shifts can also result from mutation or deletion of the small nonessential Rpb9 subunit of *S. cerevisiae* RNAPII (13–15). Although the precise mechanism by which these TFIIF or Rpb9 mutations cause upstream shifts remains unknown, biochemical analyses have shown that these mutations alter TFIIF-RNAPII interaction and that the degree of impairment in this interaction conferred by a given mutation directly correlates with the severity in the degree of upstream shifts (12).

In a previous genetic study, Hampsey and colleagues (16) identified a mutation in the *S. cerevisiae* RPB1 gene, encoding the largest subunit of RNAPII, that caused downstream shifts similar to those conferred by TFIIB mutations (16). The determination of the *S. cerevisiae* RNAPII crystal structure demonstrates that this mutation (N445S) mapped to the vicinity of the polymerase active center (17). Based upon this observation, we initiated a site-directed mutagenesis study of the active center to potentially identify residues in Rpb1 that play critical roles in the mechanism of normal start site usage. We report here the identification of two *rpb1* mutations, *rpb1-K332A* and *rpb1-R344A*, that confer altered growth properties and pronounced downstream shifts in start site utilization. Examination of the location of residues Lys-332 and Arg-344 in more recent structural determinations of a transcribing polymerase complex (18–20) demonstrates that both residues reside within the switch 2 region and are positioned within 3–5 Å of the template DNA strand immediately upstream of the location of nucleotide addition. Results from gel mobility shift assays demonstrated that wild-type and Rpb1-R344A mutant polymerase exhibited comparable activities for DNA binding and for the formation of RNAPII-DNA-RNA complexes containing a complementary 9-nucleotide RNA in the active center. In striking contrast, the mutant polymerase was severely impaired for the generation of stable RNAPII-DNA-RNA complexes containing a complementary 5-nucleotide RNA. Importantly, purified Rpb1-R344A polymerase also exhibited an increased frequency of abortive transcription at A-U base pairs at the 3’-end of the RNA-DNA hybrid in runoff transcription assays and both a downstream shift in...
start site utilization and a relative increase in the level of abortive transcripts in transcription reactions reconstituted with purified yeast general factors. We discuss a model that incorporates the role of RNA-DNA hybrid stability in the formation of productive mRNA 5′-ends and the concerted roles played by TFIIB, TFIIF, and RNAPII during this process.

EXPERIMENTAL PROCEDURES

Yeast Strains and Genetic Methods—All strains used in this study are derivatives of S288C. Strain FP294 (MATa ura3–52 trp1Δ63 rpb1Δ [p316/Rpb1 (URA3)]) contains a deletion of the chromosomal RPB1 locus and harbors the RPB1 gene on the URA3-containing vector pRS316. QuikChange site-directed mutagenesis (Stratagene) was employed to introduce amino acid substitutions into RPB1 contained on the TRP1-containing vector pRS314. The ability of the mutant plasmids to support viability was determined using the plasmid-shuffle complementation assay, and viable mutants were tested for conditional growth phenotypes as described previously (12). Complete lineages of all plasmids and strains used are available upon request.

Primer Extension Analysis of in Vivo RNA—Total RNA was isolated from yeast cultures grown in rich medium (1% yeast extract/2% peptone/2% dextrose), and ADH1 mRNA 5′-ends were mapped by primer extension as described previously (12).

RNAPII Purification and Mobility Shift Assays—Wild-type and Rpb1-R344A mutant RNAPII were purified from yeast as described previously (15). Mobility shift assays were performed as described in Ref. 15, with modifications. Reactions (20 μl) contained 20 mM HEPES-KOH, pH 7.6, 5% glycerol, 20 mM potassium acetate, 30 mM potassium chloride, 7.5 mM magnesium acetate, 2.5 mM dithiothreitol, 25 μg/ml bovine serum albumin, 0.5 pmol of the 5′-end-labeled or unlabeled DNA38, 0.2 pmol of wild-type or R344A polymerase, and 0.5 pmol of [32P]RNA9 or [32P]RNA5, as indicated in the figure legends.

Transcription Assays—For runoff transcription, RNAPII DNA [32P]RNA complexes were assembled under the conditions used in the mobility shift assays, and transcription was initiated by the addition of unlabeled NTPs to a final concentration of 50 μM. Reactions were incubated at ambient temperature for 10 min, and a portion was analyzed by denaturing polyacrylamide gel electrophoresis (24% acrylamide 12:1, 7M urea). Reconstituted reactions using purified RNAPII and general transcription factors were performed as described previously (15). For dinucleotide-primed reactions, transcription complexes were assembled for 10 min at ambient temperature. The dinucleotide was then added to a final concentration of 1 μM, and incubation continued for an additional 15 min. Transcription was then initiated by the addition of NTPs (100 μM ATP, 100 μM GTP, 100 μM UTP, 1.67 μM CTP, 111 nM [α-32P]CTP final

FIGURE 1. Mutations in the switch 2 region of Rpb1 confer altered growth properties and downstream shifts in start site utilization. A, growth properties. Cultures of the indicated strains were grown in rich medium and 10-fold serial dilutions were spotted on rich medium and incubated at the indicated temperatures for 2–3 days. B, primer extension analysis. Total RNA from the indicated strains was analyzed using an ADH1-specific primer. The numbers to the left indicate the position of the start sites, where +1 is defined as the A in the translation-initiating ATG.

FIGURE 2. Functional interactions between rpb1 switch 2 mutations and TFIIF tfg1-E346A. Growth (A) and primer extension analyses (B) were performed as described in the legend to Fig. 1. Arrows indicate the positions of enhanced upstream transcripts conferred by tfg1-E346A, and asterisks indicate the positions of enhanced downstream transcripts conferred by rpb1 mutations. WT, wild-type.
After 20 min at ambient temperature, transcription was terminated by heat inactivation at 95 °C for 3 min. Reactions were then treated for 15 min at 37 °C with 5 units of DNase I followed by treatment with 1.5 units of proteinase K for 15 min at 37 °C. A portion was analyzed by denaturing polyacrylamide gel electrophoresis (7.5% acrylamide 19:1, 7 M urea).

RESULTS

Mutations in the Switch 2 Region of the RNAPII Active Center Confer Downstream Shifts in Transcription Start Site Utilization

To identify residues within the S. cerevisiae RNAPII active center that play critical roles in start site utilization, site-directed mutagenesis was used to introduce alanine substitutions of highly conserved residues in the Rpb1 subunit. The rpb1 mutant plasmids were initially tested for their ability to support viability using the plasmid-shuffle complementation assay and strain FY294. Of the 29 plasmids tested, all were competent for supporting viability on 5-FOA medium (data not shown). When tested for growth on rich medium at varying temperatures, 27 of the mutants grew indistinguishably from the wild-type strain. These included mutants containing the rpb1 substitutions, N339A, D346A, V352A, P357A, L359A, Q363A, D408A, G410A, D411A, R412A, I413A, D414A, D414K, L415A, R416A, Y417A, S418A, K419A, R420E, L443A, R446A, V474A, Y478A, M487A, P492A, and L504A (data not shown).

The remaining two mutants, which contained the rpb1-K332A or rpb1-R344A substitutions, exhibited diminished growth at all temperatures tested (Fig. 1A). To analyze the effects of these rpb1 mutations on transcription initiation in vivo, primer extension was employed to map the transcription start sites utilized at the ADH1 promoter in the mutant strains. Compared with the wild-type strain, the rpb1-K332A and rpb1-R344A mutant polymerases have lost the ability to bind to the ADH1 promoter.
R344A mutants both displayed the classic pattern of downstream shifts in start site usage, with the rpb1-R344A allele conferring more pronounced shifts (Fig. 1B).

Examination of the location of residues Lys-332 and Arg-344 in the RNAPII crystal structure revealed that both residues reside within the switch 2 region. Switch 2 has been proposed to be relatively disordered prior to the transition to a transcribing complex but, upon the transition, adopts a more ordered structure that becomes positioned in the active center to potentially contact the RNA-DNA hybrid (17, 19). To further investigate the potential role of switch 2 in start site utilization, we tested whether the rpb1-K332A and rpb1-R344A mutations functionally interact with the tfg1-E346A mutation in the largest subunit of TFIIF, which we recently reported confers upstream shifts in start site utilization in vivo and in vitro (12). Analysis of the growth properties and in vivo initiation patterns of single and double mutant strains demonstrated that the rpb1-K332A,tfg1-E346A double mutant exhibited better growth than either the rpb1-K332A or tfg1-E346A single mutants and a partial suppression of the upstream shifts caused by tfg1-E346A (Fig. 2A; 2B, lanes 1–4). Analysis of the rpb1-R344A,tfg1-E346A double mutant demonstrated that the rpb1-R344A mutation was more effective at suppressing the upstream shifts due to tfg1-E346A, but the double mutant was not detectably healthier than either of the corresponding single mutants, presumably because of the relative persistence of the stronger downstream shifts conferred by rpb1-R344A (Fig. 2A; 2B, lanes 5 and 6). Taken together, these results indicate that the switch 2 region of S. cerevisiae RNAPII plays a functional role during start site utilization.

The Rpb1-R344A polymerase exhibits increased abortive transcription at A-U base pairs at the 3'-end of the RNA-DNA hybrid. A, runoff transcription assays using template DNA38. Reactions were incubated as in the mobility shift assays (Fig. 3B) to allow for the formation of RNAPII-DNA-[32P]RNA complexes, and transcription was initiated by the addition of unlabeled NTPs (50 μM final). After 10 min at ambient temperature, the reactions were terminated and analyzed on a denaturing polyacrylamide gel. B, runoff transcription assays using templates DNA48A- and DNA48A2. RNAPII-DNA-[32P]RNA complexes containing [32P]RNA9 and either DNA48A- (lanes 1–3) or DNA48A2 (lanes 5–7) were assembled and transcription carried out as in B. C, presented are the sequences for oligonucleotides DNA38, RNA9, RNA5, DNA48A-, and DNA48A2. Arrows indicate the locations of the 3'-ends of the major abortive transcripts. WT, wild-type.
lanes 1–3), and for the formation of RNAPII-DNA-[\(^{32}\)P]RNA complexes containing the complementary 9-nucleotide RNA in the active center (Fig. 3B, lanes 4–6). In striking contrast, the mutant polymerase was severely impaired for the generation of stable RNAPII-DNA-[\(^{32}\)P]RNA complexes containing a complementary 5-nucleotide RNA (Fig. 3B, lanes 7–9 and 7*–9*).

The Rpb1-R344A Polymerase Exhibits Increased Abortive Transcription at A-U Base Pairs at the 3’-End of the RNA-DNA Hybrid—To test the functionality of the RNAPII-DNA-[\(^{32}\)P]RNA complexes containing wild-type or switch 2 mutant polymerase, binding reactions were performed as in the mobility shift assays to allow for complex formation, and runoff transcription was then initiated by the addition of unlabeled NTPs. The results confirmed that RNAs5 and RNAs9 could be extended upon the addition of NTPs, consistent with their position in the active center (Fig. 4A). Importantly however, these results revealed that the Rpb1-R344A mutant polymerase exhibited increased abortive transcription from complexes containing either [\(^{32}\)P]RNA5 ([Fig. 4A, lanes 2–4]) or [\(^{32}\)P]RNA9 ([Fig. 4A, lanes 5–7]). The major termination products, 19 and 20 nucleotides in the reactions containing [\(^{32}\)P]RNA9, were present to a lesser extent in the reactions containing the wild-type polymerase and corresponded to transcripts that terminated with one or two consecutive uridylates at the 3’-end of the transcript (Fig. 4C). To further test whether the observed increase in abortive transcription with the mutant polymerase correlated with the presence of an A-U base pair at the 3’-end of the RNA-DNA hybrid, the runoff assays were performed using DNA template oligonucleotides that either lacked adenosines downstream of the sequence complementary to RNA9 (DNA48A-) or contained two adenosines positioned 17 nucleotides downstream (DNA48A2). The results demonstrated that the wild-type and mutant polymerase exhibited indistinguishable runoff transcription patterns from complexes containing DNA48A- ([Fig. 4B, lanes 1–3]), but that the mutant polymerase again displayed increased abortive transcription at the position of the adenosines in DNA48A2 ([Fig. 4B, lanes 5–7]). Similar results were obtained using [\(^{32}\)P]RNA5 priming of templates DNA48A- and DNA48A2 (data not shown).

The Rpb1-R344A Polymerase Confers Downstream Shifts in Start Site Utilization and Increased Abortive Initiation in Reconstituted Transcription Assays—The preceding results from the runoff transcription assays indicated that purified Rpb1-R344A polymerase was more prone to abortive transcription than wild-type RNAPII. However, because the runoff reactions utilized preformed RNAPII-DNA-[\(^{32}\)P]RNA complexes in the absence of any additional factors, reconstituted transcription assays were next performed to determine the relative activities of the mutant and wild-type polymerases in the presence of all of the yeast general factors. In the initial experiment, standard reconstituted reactions were carried out with unlabeled NTPs, and the products were analyzed by primer extension. The results showed that, compared with the reaction containing wild-type RNAPII, the Rpb1-R344A RNAPII produced a downstream shift in start site utilization and a general decrease in the overall level of transcription (Fig. 5C). To determine whether the Rpb1-R344A mutant polymerase exhibited an increased frequency of abortive initiation, reconstituted reactions were primed with the addition of an excess of the dinucleotide ApG (to prime the −37 and −27 start sites) or CpG (to prime the −91 start site). Transcription was then initiated by the addition of unlabeled ATP, GTP, UTP, and [\(^{32}\)P]CTP, and the labeled products were analyzed by denaturing polyacrylamide gel electrophoresis. In the reactions containing Rpb1-R344A RNAPII ([Fig. 5A, lanes 3 and 5]), the amount of full-length transcript was significantly lower than in the reactions containing wild-type polymerase (Fig. 5A, lanes 2 and 4). Importantly however, the

DISCUSSION

In this work, we employed site-directed mutagenesis to identify residues in the S. cerevisiae RNAPII active center that play critical roles in...
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The mechanism of transcription start site utilization. Two such residues in the switch 2 region of the Rpb1 subunit, Arg-344 and Lys-332, were identified as playing important functional roles during start site usage. This was evidenced by (i) strains containing the rpb1-R344A or rpb1-K332A mutations exhibiting conditional growth phenotypes and downstream shifts in start site utilization in vivo (Fig. 1) and (ii) the ability of the rpb1-R344A and rpb1-K332A mutations to partially suppress the upstream shifts that are conferred by the tfg1-E346A mutation in the largest subunit of TFIIF (Fig. 2).

To gain insight into the specific roles played by Arg-344 and Lys-332, we examined the location of these residues in the reported crystal structure of a transcribing S. cerevisiae polymerase complex (20). In this structure, Arg-344 is within 3.1 Å of the DNA phosphate between positions −2 and −3 relative to the site of nucleotide addition (+1), and Lys-332 is within 4.9 Å of the DNA phosphate between position −1 and +1 (Fig. 3A). The location of these residues suggested a potential role for them in stabilizing DNA binding and/or the RNA-DNA hybrid. The significance of their location in regards to the mechanism of start site utilization is further substantiated by the observation that Rpb1 Asn-445, previously identified by a mutation conferring downstream shifts similar to those reported here (16), is positioned 8–9 Å from the DNA phosphate between positions −2 and −3. Results from mobility shift assays demonstrated that the Rpb1-R344A mutant polymerase was as proficient as wild-type RNAPII for DNA38 binding and for the formation of RNAPII-DNA-RNA9 complexes but was severely impaired for the generation of stable complexes containing RNA5 (Fig. 3B). More significantly, the results from runoff transcription of RNAPII-DNA-RNA complexes revealed that the wild-type and mutant polymerases were indistinguishable in reactions containing an A-less template but that the mutant polymerase exhibited increased abortive transcription from A-containing templates at locations corresponding to the presence of one or two uridylates at the 3′-end of the transcript (Fig. 4). Results from reconstituted transcription assays using purified yeast general factors demonstrated that the Rpb1-R344A polymerase was competent for initiation, conferred a downstream shift in start site utilization, and exhibited a relative increase in the frequency of abortive initiation (Fig. 5). Taken together, these results strongly suggest that the underlying basis for the downstream shifts conferred by the Rpb1-R344A switch 2 mutant polymerase is impairment in the ability to stabilize the weaker A-U base pairs in the RNA-DNA hybrid immediately upstream of the site of nucleotide addition.

Based upon the results from this study and from previous work, we propose a model for the concerted roles played by TFIIB, TFIIF, and switch 2 during the mechanism by which a potential start site is productively utilized (Fig. 6). Shown at the top is the proposed mechanism for the formation of an mRNA 5′-end in a wild-type strain. Upon encountering a proposed Py/Aa/tPu consensus start site, RNA synthesis is initiated and the short RNA-DNA hybrid is initially stabilized by the TFIIB finger through protein-nucleic acid and/or protein-protein interactions. For the generation of a productive transcript, continued synthesis requires the complex to undergo a conformational change, perhaps mediated by TFIIF, that repositions switch 2 in the active center to stabilize the 3′-end of the RNA-DNA hybrid. This stabilization is proposed to substitute for the initial TFIIB-mediated stabilization, thereby allowing the TFIIB finger to be withdrawn from the active center to avoid steric clash with the growing RNA. For switch 2 mutants, impaired stabilization of the 3′-end of the RNA-DNA hybrid was associated with increased utilization of potential start sites encountered early during translocation (upstream shifts).
hybrid leads to an increased frequency of abortive initiation. However, we suggest that abortive initiation at a site proximal to a TATA element is followed by continued translocation of the RNAPII machinery and re-initiation, thereby increasing the levels of transcripts with 5’-ends mapping further downstream than normal (downstream shifts). Similarly, TFIIB finger mutations are proposed to impair the initial stabilization of the RNA-DNA hybrid, again leading to increased abortive initiation and re-initiation further downstream. In regards to the role of TFIIF, we suggest that a TFIIF-RNAPII interaction modulates the conformational change of the complex that is associated with the withdrawal of the TFIIB finger from the active center and the transition to a transcribing complex. Alteration of this interaction, either by TFIIF or Rpb9 mutations, results in a complex that more readily undergoes the transition. Accordingly, we propose that abortive initiation at start sites encountered early during translocation is diminished, thereby increasing the levels of transcripts with 5’-ends mapping further upstream than normal (upstream shifts). Ongoing biochemical analyses should provide additional insight into the concerted roles of switch 2, TFIIB, and TFIIF during start site utilization.

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