In vitro characterization of mutant yeast RNA polymerase II with reduced binding for elongation factor TFIIS

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ABSTRACT We have reported previously the isolation and genetic characterization of mutations in the gene encoding the largest subunit of yeast RNA polymerase II (RNAPII), which lead to 6-azauracil (6AU)-sensitive growth. It was suggested that these mutations affect the functional interaction between RNAPII and transcription-elongation factor TFIIS because the 6AU-sensitive phenotype of the mutant strains was similar to that of a strain defective in the production of TFIIS and can be suppressed by increasing the dosage of the yeast TFIIS-encoding gene, PPR2. RNAPIIs were purified and characterized from two independent 6AU-sensitive yeast mutants and from wild-type (wt) cells. In vitro, in the absence of TFIIS, the purified wt polymerase and the two mutant polymerases showed similar specific activity in polymerization, readthrough at intrinsic transcriptional arrest sites and nascent RNA cleavage. In contrast to the wt polymerase, both mutant polymerases were not stimulated by the addition of a 3-fold molar excess of TFIIS in assays of promoter-independent transcription, readthrough or cleavage. However, stimulation of the ability of the mutant RNA-PPIIs to cleave nascent RNA and to read through intrinsic arrest sites was observed at TFIIS:RNAPII molar ratios greater than 600:1. Consistent with these findings, the binding affinity of the mutant polymerases for TFIIS was found to be reduced by more than 50-fold compared with that of the wt enzyme. These studies demonstrate that TFIIS has an important role in the regulation of transcription by yeast RNAPII and identify a possible binding site for TFIIS on RNAPII.

RNA polymerase II (RNAPII) can be regulated at the transcription-elongation step as well as at the initiation step (reviewed in refs. 1 and 2). Four general transcription-elongation factors, TFIIS, TFIIF, elongin (SIIII), and ELL, have been shown in vitro to increase the overall elongation rate of transcribing RNAPII. TFIIS (SII, RAP38) stimulates incorporation of nucleotides into RNA in promoter-independent transcription (3) and facilitates the passage of RNAPII through several kinds of transcriptional blocks such as intrinsic arrest sites (4) and certain DNA-bound proteins (5). TFIIS exerts its function by binding directly to RNAPII (6, 7) and stimulating an intrinsic RNAPII endoribonuclease activity (8, 9). The other three elongation factors, TFIIF (RAP30/74), elongin, and ELL, function differently from TFIIS in that they cannot relieve RNAPII molecules that are already arrested. Instead, these factors increase the overall elongation rate of transcribing RNAPII (7, 10–14), probably by preventing arrest of RNAPII at transcriptional blocks.

Little is known about regions of RNAPII that are involved in transcript elongation and in interaction with general elongation factors. In the yeast Saccharomyces cerevisiae, deletion of the TFIIS-encoding gene, PPR2, renders cells viable but sensitive to the uracil analog, 6-azauracil (6AU) (15). Addition of 6AU to a yeast culture results in a significant depletion of intracellular GTP and UTP pools (15). Since reducing NTP concentrations in vitro decreases the elongation rate and increases pause and arrest of RNA polymerase (16–19), it has been hypothesized (15, 20) that a similar effect may be obtained in vivo by growth of yeast cells in the presence of 6AU. Thus, the 6AU-sensitivity of the PPR2 deletion mutant (ppr2A) cells may be related directly to the well-characterized TFIIS-mediated readthrough of intrinsic blocks to elongation by RNAPII in vitro. This reasoning led to the speculation that mutations resulting in 6AU-sensitive growth might also exist in regions of the subunits of RNAPII that are involved in the TFIIS-mediated function.

A genetic search of the gene, RPO21, which encodes the largest subunit of RNAPII, revealed seven mutations leading to 6AU-sensitive growth (20). All of them affected amino-acid residues that were located between the highly-conserved regions G and H of Rpo21p (20–22) and all were suppressed by elevated dosage of wild-type (wt) PPR2 (20), implicating a perturbation of TFIIS-dependent elongation. Two models were proposed to explain this result (20). First, the mutant polymerases themselves might have aberrant elongation properties that result in an increase in the number of mutant polymerase molecules stopped on the DNA template and therefore require a greater number of TFIIS molecules. Second, the alterations in the mutant polymerases may weaken the interaction between RNAPII and TFIIS, demanding more TFIIS to drive the interaction toward the formation of a normal number of active RNAPII-TFIIS complexes. To distinguish between these models, we have purified two of the seven mutant polymerases and have performed a series of in vitro assays. We report here that the alterations in the mutant RNAPIIs weaken the interaction between the enzymes and TFIIS.

MATERIALS AND METHODS

Purification of Proteins. Recombinant yeast TFIIS protein, tagged at the N terminus with six histidine residues (to facilitate purification) and a five-amino acid recognition element for bovine-heart-muscle kinase (to facilitate labeling; ref. 23), was purified to virtual homogeneity from Escherichia coli cells essentially as described (24). No significant differences were detected in the stimulation of readthrough and cleavage activities of RNAPII between this preparation of recombinant TFIIS protein and a variety of yeast TFIIS proteins described previously (24). Wt and mutant RNAPIIs were purified to near homogeneity from yeast strains expressing wt RNAPII, rpo21-18 (I1237TRARV), or rpo21-24 (E1230K) mutant RNAPII (20) as described (25). Protein concentrations were determined by the method of Bradford (26).

Abbreviations: RNAPII, eukaryotic RNA polymerase II; 6AU, 6-azauracil; ts, temperature sensitive; wt, wild type.

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In Vitro Transcription Assays and RNase-Cleavage Assays. Promoter-independent transcription assays using poly(rC) or denatured calf-thymus DNA as a template were performed as described (7, 25). TFIIIS-stimulated readthrough assays and ternary-complex cleavage assays using poly(dC)-tailed human histone H3.3 DNA as a template were performed as described (24).

Briefly, RNA transcripts were pulse-labeled at the 5’ ends by incubating in the presence of 33.3 nM [γ-32P]ATP [10.0 μCi, 3000 Ci/nmol (1 Ci = 37 GBq), DuPont] and 0.8 mM each ATP, GTP, and UTP for 75 sec, followed by elongating to the three intrinsic blocks in the presence of 0.1 mM unlabeled CTP and 100 μg/ml heparin (to prevent reinitiation) for 75 sec. Most of the nascent transcripts derived from this template were displaced from the template DNA strand (27). Ternary complexes stopped at the intrinsic arrest sites were treated in two different ways. The readthrough assay was done as follows. Yeast TFIIIS was added, transcription was allowed to proceed for the designated intervals, and the resulting transcripts were collected by ethanol precipitation and were resolved by electrophoresis on a 7% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 8.3 M urea, 2 mM EDTA, and buffered with 89 mM Tris pH 8.3, 50 mM ZnSO4, and 1% glycerol (pH 8.3), at 4°C. The amount of TFIIIS–RNAPII binary complexes was determined using the Molecular Dynamics PhosphorImager with 32P-labeled TFIIIS blotted on to filter paper as a standard. The apparent dissociation constant (k_d) was determined using Scatchard analysis.

RESULTS

Specific Polymerization Activities of wt and Mutant Polymerases on a Poly(rC) Template Are Comparable. The polymerization activities of the wt and mutant polymerases were compared on a poly(rC) template. On this template, purified RNAPII incorporates GMP in a template-dependent manner (29) under various conditions and produces high radioactive signals (at least 10-fold higher than background) that represent newly synthesized RNAs. Under normal conditions (1 mM GTP and 30°C), specific polymerization activities of wt and mutant polymerases were comparable (Fig. 1A and B). The activities were estimated to be in the range of 500–620 nmol of nucleotide incorporated per min per mg of protein at 30°C. Since the mutant strains used in this study are 6AU-sensitive (which is probably related to the depletion of intracellular GTP pool by 6AU; ref. 15) as well as temperature-sensitive for growth, the purified polymerases were also assayed at reduced GTP concentration (20 μM; Fig. 1C) and at elevated reaction temperatures (42°C; Fig. 1D). Under these two sets of conditions, the polymerization activities of both wt and mutant
polymerases were decreased to a similar extent. Therefore, the two rpo21 mutations (I1237TRARV and E1230K) do not exacerbate the negative effect of nucleotide-substrate limitation or heat on the polymerization activity of RNAPII.

Polymerization Activity of Mutant RNA Polymerases Is Insensitive to Stimulation by TFIIS. Purified RNAPII transcribes denatured duplex DNA at a low efficiency, which can be stimulated 2- to 10-fold by TFIIS (6). Wt and mutant RNAPII preparations were tested for this property by using denatured calf thymus DNA as a template (Fig. 2). In the absence of TFIIS, the activities of wt and mutant polymerases were indistinguishable. Maximum stimulation of wt RNAPII by TFIIS was obtained at a TFIIS/RNAPII molar ratio of ~1.5:1. In contrast, at the same TFIIS/RNAPII molar ratio, or slightly higher, no significant stimulation of the mutant enzymes was detected. At a molar ratio of at least 75:1, stimulation by TFIIS was observed. Even at the highest molar ratio tested (225:1), only ~45% of the maximal stimulation of wt polymerase was achieved for the mutant enzymes.

High Concentrations of TFIIS Are Required by the Mutant RNA Polymerases to Approach Levels of wt Readthrough of Intrinsic Arrest Sites on Human Histone H3.3 DNA. Readthrough activity of wt and mutant polymerases at intrinsic blocks to elongation were compared in an assay using poly(dC)-tailed human histone H3.3 gene DNA as a template. It has been shown that purified yeast RNAPII recognizes and stops at the three intrinsic transcriptional arrest sites on the human histone H3.3 DNA but does not terminate (24, 25), and that yeast TFIIS can stimulate RNAPII to read through these sites (24).

Two observations were noteworthy at the outset. First, wt and mutant polymerases stop at the identical intrinsic arrest sites in the human histone H3.3 gene (Fig. 3 A, lanes 1, 8, and 15); no additional arrested complexes were observed in the case of the two mutant polymerases. Second, comparable percentages of wt and mutant polymerase molecules were arrested at each of the three sites. For example, the proportion of molecules arrested at the T1a site following a 30-min incubation was 64% for the wt enzyme and 58% for both mutant enzymes (Fig. 3 A, lanes 1, 8, and 15). The time course of the readthrough activity of the wt and mutant enzymes at the three intrinsic arrest sites was examined. In the absence of TFIIS, wt and mutant enzymes behaved indistinguishably following reaction times of 5, 10, and 30 min (data not shown). These observations, as well as those presented above, indicate that the two rpo21 mutations do not affect the polymerization activity of RNAPII on different templates. Thus, the suggestion that the mutant enzymes are more prone to arrest in vivo (see above and ref. 20) is not likely to be correct.
A striking difference was observed between the wt and the two mutant polymerases in the presence of a 10-fold molar excess of TFIIS (Fig. 3A, lanes 2, 9, and 16). The fraction of arrested wt RNAPII was reduced to 20%. In contrast, at this molar ratio, the fraction of arrested mutant RNAPIIs remained unchanged, suggesting that at this level TFIIS does not stimulate mutant RNAPIIs to read through the arrest sites.

In an effort to mimic the in vivo observation that the rpo21 mutants can be suppressed by overproduction of TFIIS (20), TFIIS-stimulated readthrough activity of the two mutant polymerases was tested at greatly elevated TFIIS/RNAPII molar ratios. As shown in Fig. 3, only massive addition of TFIIS to the mutant polymerases (over 600:1, TFIIS/RNAPII) resulted in readthrough comparable to that of the wt polymerase.

Stimulation of Intrinsic RNase Activity in the Mutant RNA Polymerases Requires High Concentrations of TFIIS. The RNase activity (both intrinsic and TFIIS-stimulated) of wt and mutant RNAPII preparations was compared. Ternary elongation complexes containing RNA polymerase, DNA template and nascent RNA transcript arrested at the T1a, T1b, and TII sites and run-off transcripts were isolated free of nucleotides by centrifugal gel filtration (24). In the absence of TFIIS, incubation of the ternary complexes for as long as 2 hr revealed only very low levels of intrinsic RNase activity (Fig. 4A and B). Following a 2-hr incubation period, nucleotides were added to the complexes to allow for RNA-chain extension. The resulting transcripts were resolved by gel electrophoresis and visualized by autoradiography. The mobilities of the run off (RO), the T1a, the TII and the cleaved product (CP) are indicated on the left.

In the presence of a 3-fold molar excess of TFIIS, incubation for 0.2, 1, and 2 hr of ternary complexes containing wt RNAPII resulted in cleavage of a majority of the transcripts with 3' ends at the T1a site (Fig. 4A, lanes 7–9). In contrast, incubation of ternary complexes containing the Rpo21-24 (E1230K) mutant polymerase (at 3:1, TFIIS/RNAPII) resulted in no more cleavage than was seen in the absence of TFIIS (Fig. 4B, compare lanes 7–9 to lanes 2–4). Therefore, the intrinsic RNase activity of the two mutant enzyme complexes is not stimulated by addition of this amount of TFIIS protein.

When the mutant enzymes were incubated in the presence of a 600-fold molar excess of TFIIS (Fig. 4B, lanes 12–14), the RNA-cleavage ability was stimulated to a level that was similar to that shown by the wt enzyme in the presence of a 3-fold molar excess of TFIIS (compare Fig. 4B, lanes 12–14 to Fig. 4A, lanes 7–9). Therefore, provided sufficient TFIIS protein is present, the stimulated cleavage activity of the two mutant polymerases can approach normal levels.
We have observed that two mutations, which lead to 6AU-sensitive growth and affect amino-acid residues that are located between the highly conserved regions G and H of the largest subunit of RNAPII (Rpo21p; ref. 20), reduce the binding affinity of the mutant enzymes for TFII S by more than 50-fold. As a consequence, very large amounts of TFII S protein are required to stimulate the arrested mutant enzymes in cleavage of nascent RNAs and readthrough of intrinsic arrest sites.

These in vitro observations offer a plausible explanation for the 6AU-sensitive phenotype of the rpo21 mutants and the suppression of the 6AU sensitivity by increased gene dosage of PPR2. Addition of 6AU to a yeast culture results in more than a 10-fold depletion of the intracellular GTP pool and a 2- to 3-fold depletion of the UTP pool (15). Several investigators have observed that the efficiency with which elongating RNAP becomes paused at intrinsic pausing sites depends directly on the elongation rate of transcribing RNA polymerase and to NTP substrate concentration; a reduction in the concentration of one or all of the four NTPs enhances arrest of RNA polymerase (16–19). Therefore, the ultimate effect of 6AU on growing cells might be an increase in the number of transcribing RNAPII molecules that arrest or pause on the DNA template. When this occurs, cell growth becomes dependent on TFII S. With the help of TFII S, wt RNAPII might be able to complete a sufficient number of transcripts to permit growth in the presence of 6AU, whereas the mutant enzymes might not because the interactions between TFII S and the mutant enzymes are weakened. An increase in the gene dosage of PPR2 leads to increased production of yeast TFII S protein and an elevated intracellular concentration of TFII S. By driving the assembly of a sufficient number of active RNAPII–TFII S complexes, an elevated PPR2 gene dosage compensates for the reduced binding affinity of mutant RNAPII for TFII S and allows growth.

Six of the seven 6AU-sensitive rpo21 mutants (including the two described in this study, E1230K and I1237TRARV) are also temperature-sensitive (ts) for growth (20), which might also be related to reduced binding of the mutant enzymes for TFII S. We have observed that the stopping efficiency of wt RNAPII at intrinsic arrest sites is increased substantially at elevated temperature (unpublished data). For example, at 38°C, the fraction of arrested wt RNAPII at the first and...
weaker arrest site in the human histone H3.3 gene, TII, increases to ~50% from the 15–25% seen at 30°C (data not shown). Therefore, raising the temperature may achieve a similar effect as that generated by adding 6AU to yeast growth medium—i.e., more transcribing RNAPII molecules may stop on the DNA template, rendering cell growth dependent on TFIIS function. However, since the ts phenotype of rpo21 mutants cannot be suppressed by overproduction of TFIIS (20), one would have to assume that at a high temperature, the interactions between TFIIS and the mutant enzymes are so weak that they cannot be restored. Alternatively, alterations in the G–H region of Rpo21p that confer 6AU-sensitivity could also affect other RNAPII functions, such as promoter-dependent initiation, termination, or assembly of the enzyme. Two observations support this possibility. First, the ppr2Δ mutant strain has a near wt growth rate at the permissive temperature (30), whereas one of the 6AU-sensitive rpo21 strains, rpo21-7 (T1141TSSSS), has a very slow growth rate at 30°C (data not shown). Therefore, raising the temperature may achieve a similar effect as that generated by adding 6AU to yeast growth medium—i.e., more transcribing RNAPII molecules may stop in the G–H region of Rpo21p. Second, at least one ts mutation in the G–H region of Rpo21p (C1240Y) has been shown to affect the assembly of RNAPII at a nonpermissive temperature (30).

All seven of the rpo21 mutations leading to 6AU-sensitive growth affect amino acid residues that are clustered between highly conserved regions G and H of Rpo21p (20–22). We suggest that our rpo21 mutations might weaken the interaction between TFIIS and RNAPII by altering a site on RNAPII that is involved in direct interaction between TFIIS and RNAPII. The nature of the rpo21-24 (E1230K), which changes a glutamic acid to a lysine, suggests that a portion of this TFIIS binding site may be negatively charged. TFIIS might be expected to bind to a negatively charged surface because TFIIH is very basic. The RNAPII/TFIIS interaction would thus be predicted to be partly ionic in nature, which is consistent with the observations that the RNAPII/TFIIS interaction is known to be disrupted by low to moderate salt concentrations. However, we cannot rule out the possibility that the mutations induce conformational changes that reduce indirectly the binding of TFIIS to RNAPII.

Conserved region B of Rpo21p has also been implicated in TFIIS binding (31); a fusion protein containing a fragment of region B, as well as a monoclonal antibody thought to be directed to region B, when added at high concentrations partially inhibit TFIIS-stimulated transcription. However, the interpretation of these experiments is not entirely clear, since no direct interaction between the polymerase domain and TFIIS was measured and the inhibition of TFIIS activity was not reported to be overcome by the addition of excess TFIIH. Nevertheless, if both conserved regions B and G–H do play a role in TFIIS interaction, these two domains might be in close proximity in the RNA polymerase II structure.

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