Mutations in the Second Largest Subunit of RNA Polymerase II Cause 6-Azauracil Sensitivity in Yeast and Increased Transcriptional Arrest in Vitro*

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Yeast RNA polymerase II enzymes containing single amino acid substitutions in the second largest subunit were analyzed in vitro for elongation-related defects. Mutants were chosen for analysis based on their ability to render yeast cells sensitive to growth on medium containing 6-azauracil. RNA polymerase II purified from three different 6-azauracil-sensitive yeast strains displayed increased arrest at well characterized arrest sites in vitro. The extent of this defect did not correlate with sensitivity to growth in the presence of 6-azauracil. The most severe effect resulted from mutation rpb2-10 (P1018S), which occurs in region H, a domain highly conserved between prokaryotic and eukaryotic RNA polymerases that is associated with nucleotide binding. The average elongation rate of this mutant enzyme is also slower than wild type. We suggest that the slowed elongation rate and an increase in dwell time of elongating pol II leads to rpb2-10's arrest-prone phenotype. This mutant enzyme can respond to SII for transcriptional read-through and carry out SII-activated nascent RNA cleavage.

RNA polymerase II (pol II) can encounter a variety of transcriptional blocks during the elongation phase of RNA synthesis. These include DNA sequences (reviewed in Kane, 1994; Reines, 1994), DNA bound proteins (Deuschle et al., 1990; Izban and Luse, 1993; Kuhn et al., 1990; Reines and Mote, 1993), DNA binding drugs (Mote et al., 1994), and covalent modifications due to DNA damage (Donahue et al., 1992). In vitro studies have identified mechanisms by which elongating pol II can overcome some of these obstacles. Intrinsic DNA sequences that cause pol II to become arrested have been most extensively investigated and are frequently employed as a model in studies of transcriptional arrest.

Stably arrested pol II can be reactivated for RNA chain extension by elongation factor SII. SII binds the enzyme and activates a ribonuclease activity thought to reside within pol II (reviewed in Reines 1994). This ribonuclease removes a small number of nucleotides from the 3' end of the nascent RNA prior to its re-extension through the blockage (Izban and Luse, 1993a, 1993b; Gu and Reines, 1995b) in a reiterative cleavage and resynthesis process, allowing a pol II molecule multiple attempts at chain extension through an obstacle (Gu et al., 1993; Guo and Price, 1993; Reines, 1994).

The extent of arrest can be reduced or prevented by increasing pol II's rate of nucleotide addition to the nascent RNA chain, which decreases the dwell time of the enzyme at each template position (Bengal et al., 1991; Izban and Luse, 1992; Gu and Reines, 1995a). pol II's elongation rate can also be influenced by ammonium ions (Izban and Luse, 1991, 1992) and elongation factors TFIIF (Flores et al., 1989; Price et al., 1989; Bengal et al., 1991; Izban and Luse, 1992) and SIII (elogin; Aso et al., 1995). Conversely, when nucleotide addition is slowed and dwell time is lengthened by a reduction in nucleoside triphosphate concentration, arrest efficiency increases (Wiest and Hawley, 1990; Wiest et al., 1992; Gu and Reines, 1995a). Similarly, reduced in vitro elongation rate has been correlated with increased termination by other RNA polymerases. "Slow" E. coli RNA polymerase mutants are termination-prone (Ij and Gross, 1991), and termination-prone eukaryotic RNA polymerase III mutants containing amino acid substitutions in the second largest subunit are reported to have slowed elongation rates (Shaaban et al., 1995).

Less is known about the presence and nature of transcription elongation blockages in vivo and of the function of elongation factors in overcoming potential transcriptional blocks. In yeast, disruption of the gene encoding elongation factor SII renders cells sensitive to growth in the presence of 6-azauracil (6AU) (Exinger and Lacroute, 1992; Nakanishi et al., 1992), which causes a 2–3-fold reduction in cellular UTP levels and a 10-fold reduction in GTP levels (Exinger and Lacroute, 1992). Yeast lacking SII are also sensitive to mycophenolic acid, another inhibitor of NTP synthesis (Exinger and Lacroute, 1992).

Mutations of the largest subunit of pol II (RPB1) also confer growth sensitivity to 6AU (Archambault et al., 1992). These mutations are clustered within a 470-base pair region conserved among eukaryotes. These are the only reported mutations in the 12-subunit pol II enzyme that confer 6AU sensitivity. Interestingly, the 6AU sensitivity caused by each of these mutant alleles can be complemented by overexpression of SII (Archambault et al., 1992), providing in vivo evidence for a role of SII in pol II transcription and suggesting that depression of NTP levels increases the reliance of the enzyme upon SII. These findings have also suggested that 6AU sensitivity might serve as a bioassay for mutations in components of the elongation machinery, although a direct demonstration that 6AU-sensitive RPB1 alleles generate an elongation-defective enzyme has not been reported.

Several lines of evidence implicate the second largest subunit of pol II (RPB2) in transcript elongation, including binding of nucleotide substrates (Treich et al., 1992) and RNA products (Bartholomew et al., 1986) during RNA synthesis. Genetic ex-
Experiments reveal that homology region H, which is strongly conserved among all organisms, is important for the enzyme's function (Scafe et al., 1990a).

The implied role of RPB2 in transcript elongation and potential applicability of 6AU as a screen for mutant alleles that contribute to transcriptional elongation defects prompted us to test the enzymatic activity of 6AU-sensitive RPB2 mutants and assess the relevance of the 6AU-sensitive phenotype with respect to distinct, elongation-related activities in vitro. Here, we report that three mutations in RPB2 that confer 6AU sensitivity to haploid yeast strains display an increased propensity to become arrested at intrinsic DNA sequences in vitro, a trait likely attributable to their reduced rate of factor-independent nucleotide addition.

MATERIALS AND METHODS

Yeast Strains—Haploid yeast strains Z422, Z425, and Z428 contain mutant alleles rpb2-4, rpb2-7, and rpb2-10, respectively, in an isogenic background (MATa his3Δ200 leu-3, 112 ura3-52 rpb2Δ297::HIS3 [URA3, rpb2-x], where x represents the specific mutant allele). Strain Z480 (MATa his3Δ200 leu-2, 3, 112 ura3-52 rpb2Δ297::HIS3 [LEU2, rpb 2-1]) expresses HA epitope-tagged wild type RPB2, and strain Z24 (MATa his3Δ200 leu-2, 3, 112 ura3-52 rpb2Δ297::HIS3 [URA3, RPB2]) expresses the untagged wild type enzyme. These strains were kindly provided by Dr. R. A. Young (MIT).

Enzyme Purification—Strains Z422, Z425, Z428, and Z480 were independently grown, harvested, and lysed by the method of Edwards et al. (1990). Briefly, cells grown in YPD medium (1% yeast extract/2% bacto-peptone/2% glucose) were washed once in cold water, resuspended in 1.2 volumes of storage buffer (0.25 M Tris-HCl, pH 7.9, 5 mM EDTA, 2.5 mM DTT, 10 mM sodium pyrophosphate, 5% dimethyl sulfoxide, 50% glycerol), and frozen at −80°C. Thawed cells were mixed at 4°C with 1.25 volumes glass beads and disrupted by fifteen 30-s bursts of a bead beater (Biospec Products, Bartlesville, OK) with 90 s of cooling between bursts. The lysate was diluted into 0.6 volumes of buffer A (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 mM DTT, 10 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol and protease inhibitors (per liter: 10 mg of aprotinin, 320 mg of benzamidine, 1 mg of pepstatin, 10 mg of leupeptin, 5 mg of 1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, 174 mg of phenylmethylsulfonyl fluoride). Glass beads and cell debris were removed by centrifugation, and the supernatant was applied to a 40-ml heparin-Sepharose CL-6B column (Pharmacia Biotech Inc.) (Edwards et al., 1990). The column was washed with buffer A containing 0.1 M KCl and eluted with buffer A containing 0.6 M KCl. Peak protein fractions were pooled, precipitated with 50% (w/v) saturated ammonium sulfate, and dialyzed versus buffer B containing 0.1 M KCl.

This protein was applied to a DEAE 5-PW column (Bio-Rad, 75 × 7.5 mm). Bound material was eluted with a 9-ml gradient from 0.1-0.5 M KCl (Conaway and Conaway, 1990). Fractions were assayed by Western blots using monoclonal antibody 8WG16 (Thompson et al., 1989). Specific activity of peak fractions was determined in a nonspecific transcription assay (Sawadogo et al., 1980). Nucleotide incorporation was greater than 90% ρ-amanitin-sensitive (100 μg/ml), and specific activity ranged from 18–42 units/mg protein (1 unit = 1 nmol nucleotide incorporated per minute). In assays using tailed templates, pol II fractions produced transcripts that were completely sensitive to ρ-amanitin.2 We estimate a purity of 10–20% by specific activity and silver staining of SDS gels.

Expression and Purification of Recombinant Yeast SII—Yeast SII was expressed from pYSE1, a gift from S. Natori (University of Tokyo, W. Powell, unpublished data.)
Cells were harvested by centrifugation at 4,000 $g$ for 15 min, washed in 25 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 1 mM $\beta$-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium metabisulfite, and stored at $-80^\circ$C. After sonication the lysate was centrifuged at 12,000 $g$ for 5 min, and the supernatant was applied to phosphocellulose (Whatman P-11) equilibrated in 20 mM Hepes, pH 7.9, 20% glycerol, 1 mM EDTA, 10 mM $\beta$-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium metabisulfite, 0.33 M KCl. Bound material was eluted with this buffer containing 0.6M KCl. Peak protein fractions were collected, precipitated with 60% (w/v) ammonium sulfate, centrifuged at 35,000 $g$ for 30 min, dissolved in HD buffer (100 mM Hepes, pH 7.9, 0.1 mM DTT), dialyzed versus HD buffer plus 1.5M ammonium sulfate, and applied to a phenyl-5PW column (Bio-Rad; 75 $\times$ 7.5 mm).

SII was eluted with HD and a 36-ml linear gradient from 1.5M to 0M ammonium sulfate. Peak fractions were pooled and dialyzed in 40 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM DTT, and 0.1M KCl and applied to a SP-5PW column (Bio-Rad; 75 $\times$ 7.5 mm). Bound material was eluted with this buffer and a continuous gradient from 0.1 to 0.55 M KCl (Conaway et al., 1996). The peak fraction of transcript cleavage and read-through activity (Conaway et al., 1996) was used in all subsequent experiments. This SII appeared homogeneous as judged by silver-stained SDS-polyacrylamide gel electrophoresis.

**In Vitro Transcription**—Transcription reactions employed 3'-deoxy-cytidine-tailed DNA (Kadesch and Chamberlin, 1982) derived from the plasmid pUCH3D1. This plasmid contains a $Sma$-I–$Eco$-RI fragment from the human histone H3.3 gene, which contains three well characterized arrest sites (Reines et al., 1987) cloned into similarly cut pUC18. pUCH3D1 was linearized with Smal, tailed with dCTP and terminal deoxynucleotidyl transferase (Ratliff Biochemicals, Los Alamos, NM), and digested with XbaI and XhoI. The resulting tailed template yields a run-off transcript of 400 nucleotides and arrested transcripts of 150 (site II), 190 (site Ib), and 210 nucleotides (site Ia).

To facilitate comparison of these assays with those performed in promoter-driven transcription, we used the transcription conditions described for a rat liver reconstituted system (Reines et al., 1987). pol II (0.3 $\mu$g of protein) was added to 75 ng of DNA in 20 $\mu$l of total volume of 20 mM Hepes, pH 7.9, 20 mM Tris, pH 7.9, 2% (w/v) polyvinyl alcohol, 0.4 mg/ml bovine serum albumin, 12 units of RNasin (Promega), 150 mM KCl, 2 mM DTT. After 30 min at 28°C, the reaction was diluted using the same buffer lacking KCl to a final KCl concentration of 60 mM. After an additional 20 min at 28°C, MgCl$_2$, ATP, GTP and (a-$^{32}$P)CTP (3000 Ci/mmol) were added in 6 $\mu$l to final concentrations of 7, 20

**Fig. 3. SII-mediated read-through by arrested elongation complexes.** Arrested ternary complexes were assembled by extending a 16-nucleotide pulse-labeled transcript for 20 min in the presence of 800 $\mu$M ATP, UTP, and GTP plus 100 $\mu$C CTP (lanes 0). Arrested complexes were immunoprecipitated, washed, and treated with SII (approximately 30-fold molar excess), MgCl$_2$, and 800 $\mu$M of ATP, UTP, GTP, and CTP. Aliquots were stopped at indicated times. A, wild type; B, rpb2-10; C, rpb2-7; D, rpb2-4. Positions of arrested (Ia and II) and run-off length transcripts are indicated at the right of each panel; positions of RNA markers (540, 420, 380, and 260 nucleotides) are indicated by arrowheads on the left. RO, run-off.

**Fig. 4. Kineticsof elongation through arrest sites by wild type (A) and rpb2-10 (B) pol II.** Transcription reactions were performed as described in the presence or the absence of SII (approximately 150-fold molar excess). Aliquots were stopped at the indicated times. Arrested and run-off length transcripts are identified by the arrows at right. Positions of transcripts are as indicated in the legend to Fig. 3. RO, run-off.
m, 20 μM, and 0.6 μM, respectively, resulting in a transcript 16 nucleotides long, because the first uridine appears at position 17. Chain extension proceeds in the presence of heparin (10 μg/ml) and ATP, UTP, GTP, and CTP to synthesize run-off and arrested RNAs. As indicated, ternary complexes were immunoprecipitated with anti-RNA monoclonal antibody (Eilat et al., 1982, Reines, 1991). 4 μg of IgG were added per 60 μl of reaction. Complexes were collected after incubation with fixed Staphylococcus aureus cells and centrifugation for 2 min in a microcentrifuge, washed twice in reaction buffer, resuspended in 55 μl, and treated as described in the text. Reactions were stopped by the addition of 0.2M Tris-HCl, pH 7.5, 25 mM EDTA, 0.3M NaCl, 2% (w/v) SDS. S. aureus was removed by centrifugation, protein was digested with proteinase K (0.4 mg/ml), and RNA was isolated by ethanol precipitation.

Where indicated, pUC18 was tailed at the SmaI site and cut with PstI as described, allowing production of a run-off transcript of approximately 2660 nucleotides (Dedrick et al., 1987). Incubations were as described above; however, RNA was pulse-labeled for 1 min at 21°C in the presence of 20 μM each of ATP, UTP, and GTP and 10 μCi of [α-32P]CTP (3000 Ci/mmol) per reaction followed by a chase with 800 μM ATP, UTP, GTP, and CTP. RNA was subjected to electrophoresis on 7 or 4% polyacrylamide (19:1, acrylamide:bisacrylamide) gels, which were dried and subjected to autoradiography and PhosphorImager analysis.

Quantitation of Transcripts in Polyacrylamide Gels—Radioactivity was quantitated using a Molecular Dynamics PhosphorImager system. Radiolabeled RNAs with 3′ ends at sites II, Ib, and Ia, and run-off lengths were identified and the relative radioactivity in each transcript was determined for each time point. Percent read-through at a site was calculated by dividing the phosphorimaging units at that site by the total units at and downstream of that site. Thus, percent read-through at sites Ia + Ib = Ia + Ib (Ia + Ib + run-off); percent read-through at site II = II/I (Ia + Ib + RO). Note that in several cases, transcripts at sites Ia and Ib were measured as a single entity due to the difficulty in resolving their positions in these gels. Background radioactivity was measured in an area of each lane that did not contain transcripts and appropriately subtracted from each value for a specific transcript.

For elongation on pUC18, the rate of accumulation of the maximal level of run-off transcripts (or transcripts 980 nucleotides or larger) was determined by dividing the radioactivity in the run-off length band (or portion of lane containing transcripts of 980 nucleotides or larger) by the average total radioactivity found in the lanes of a gel. To verify that the amount of radiolabeled CTP incorporated into transcripts did not increase with chase time in these reactions, nucleotide incorporation was measured as described (Schwartz et al., 1974). Equal quantities of radioactivity were incorporated at each time point and loaded onto the gels.

RESULTS

Point Mutations in RPB2 Cause Sensitivity to 6-Azauracil—Three previously isolated alleles of RPB2 (Fig. 1), in addition to their reported phenotypes of cold sensitivity, heat sensitivity, and/or inositol auxotrophy (Scafe et al., 1990a, 1990b), cause sensitivity of yeast to growth in the presence of 6AU. Yeast bearing rpb2-7 are most sensitive, followed by rpb2-4 and rpb2-10 (Fig. 2). rpb2-7 also displayed measurable sensitivity to mycophenolic acid (45 μg/ml). The stronger effect of 6AU on cell growth might be expected because mycophenolic acid depresses only the GTP pool, whereas 6AU decreases the cellular levels of both UTP and GTP (Exinger and Lacroute, 1992).

 Arrest and Read-through of Mutant pol II—Because 6AU sensitivity may reflect a defect in transcriptional elongation (Archambault et al., 1992; Exinger and Lacroute, 1992), we examined the ability of each mutant enzyme to overcome arrest in a region of a human histone gene (Reines et al., 1987). Yeast pol II becomes arrested at three sites in this gene called Ia, Ib, and II (Christie et al., 1994). Arrested complexes were assembled on poly(dC)-tailed templates, which permit transcription in the absence of basal initiation factors, and isolated by im-

3 N. Hannett and R. A. Young, personal communication.
munoprecipitation with an anti-RNA antibody. Increased arrest efficiencies were observed for the mutants in the absence of SII (Fig. 3A, lane 0, versus 3B, C, and D, lanes 0). Note that in these single-round transcription assays, the extent of readthrough plateaus and remains constant with extended incubation time, consistent with previously published reports of mammalian and yeast pol II on tailed templates (Reines et al., 1987; Christie et al., 1994; see Fig. 5). After an initial 20-min incubation, 25% of transcripts in the wild type reactions had reached run-off length (Fig. 3A, lane 0), compared with 5% for rpb2–10 (Fig. 3B, lane 0), 17% for rpb2–4 (Fig. 3C, lane 0), and 20% for rpb2–7 pol II (Fig. 3D, lane 0). We examined the ability of each enzyme to respond to SII, which enables complete read-through of this site for the wild type mammalian and yeast enzymes (Siva Raman et al., 1990; Christie et al., 1994), over an additional 30-min incubation. The isolated, arrested complexes were treated with purified recombinant yeast SII and all four nucleotides. All mutants were able to read-through arrest sites, but the region H mutants did so more slowly than wild type enzyme (compare Fig. 3A–D, lanes 50–30). rpb2–10 pol II cleared the arrest sites most slowly (38% of all transcripts at run-off after 30 min), and rpb2–4 was detectably slowed (66%), whereas rpb2–7 had accumulated an equal proportion to the wild type enzyme by the final time point (both 82%). Because rpb2–10 pol II exhibited the most severe defect in these assays, this mutant enzyme became the focus of further analysis.

Kinetics of Transcript Accumulation from Early Stage Elongation in the Presence and Absence of SII—In order to observe the kinetics of accumulation of arrested and run-off RNAs at earlier time points in transcript elongation, we examined the time course of a single round of elongation of a pulse-labeled, 16-nucleotide transcript by wild type and rpb2–10 pol II (Fig. 4; quantitation in Fig. 5). In the absence of SII, rpb2–10 pol II has a 2.6-fold poorer read-through efficiency at sites Ia and Ib than does wild type (15% versus 39%; Fig. 5, upper left). It is also defective in read-through at site II (65% versus 85%; Fig. 5, upper right). In the presence of SII, the wild type enzyme reaches maximal read-through of sites Ia and Ib within 5 min, whereas the rpb2–10 enzyme reads through these arrest elements much more slowly, reaching maximal levels of read-through only after 30 min (Fig. 5, lower panels). SII enables wild type enzyme to read-through site II with near total efficiency within 1 min, whereas rpb2–10 pol II has done so by 5 min. In summary, rpb2–10 pol II becomes arrested with greater frequency in the absence of SII compared with wild type. When SII is included in the reaction, rpb2–10 pol II can read through arrest sites as well as wild type, but at a reduced rate.

Nucleotide Concentration Affects Read-through Efficiency of Mutant and Wild Type pol II—Previous work has shown that transcriptional arrest by mammalian pol II is related to the dwell time at an arrest site and therefore the concentration of the next nucleoside triphosphate to be incorporated (Wiest and Hawley, 1990; Wiest et al., 1992; Gu and Reines, 1995a). We tested the CTP dependence of arrest by wild type and rpb2–10 enzymes because CTP is the next nucleotide following sites Ia and II. As shown previously for wild type rat RNA pol II transcribing from a promoter (Gu and Reines, 1995a), we find a strong dependence of arrest upon CTP concentration for both rpb2–10 and wild type yeast pol IIIs. At 25 μM CTP, the read-through ability of wild type enzyme was comparable with the indicated concentration of CTP. Positions of transcripts are as indicated in the legend to Fig. 3B. Quantitation of data in A. Percent read-through was determined using a PhosphorImager as described in the text. Sites Ia and Ib are indicated by closed circles; site II is indicated by open circles. RO, run-off.
read-through efficiency by rpb2–10 pol II at saturating CTP levels (approximately 21% for sites Ia and Ib and 50% for site II) (Fig. 6A; quantitation in Fig. 6B).

rpb2–10 pol II Has a Slower Average Rate of Elongation—To compare elongation rates of wild type and mutant enzymes in the absence of arrest sites, we prepared linearized pUC18 as a poly(dC)-tailed template (Dedrick et al., 1987). The pUC18 template represents perhaps the best opportunity among naturally occurring (nonhomopolymer) DNA sequences to gauge factor-independent transcription over an extended distance (2.6 kilobase pairs), because it is largely free of known arrest sites, although one termination site has been described at +980 (Dedrick et al., 1987). In these single-round transcription reactions, equimolar quantities of pol II complexes were withdrawn from the reaction at each time point as measured by incorporation of radiolabeled nucleotide substrate. At early time points the apparent absence of radioactivity in the gel lanes results from smaller transcripts that have migrated beyond the end of the gel. These experiments revealed that wild type pol II reached a maximal percentage of full-length 2.6-kilobase transcripts within 20 min and was half-maximal in under 5 min (Fig. 7, A and B). By comparison, extrapolation of the rpb2–10 curve in Fig. 7B indicated that this mutant enzyme required 44 min to reach a similar level of run-off transcripts and 17 min to reach the half maximal level. This represents a reduction in the overall chain elongation rate of the mutant enzyme by 2–4-fold.

In a separate analysis of the same data, we compared the enzymes’ abilities to extend transcripts to or past the termination site at position 980 to exclude the possibility of its differential influence on the two enzymes. This analysis reveals a 2-fold difference in the time to reach half-maximal and maximal levels of accumulation of transcripts of at least 980 nucleotides (Fig. 7C).

These relative differences correspond to the different specific activity measurements for each enzyme in traditional nonspecific transcription assays on a poly(rC) template (Ruet et al., 1978). rpb2–10 enzyme had a 2.3-fold lower specific activity than wild type pol II (18 units/mg protein versus 42 units/mg; 1 unit = 1 nmol GTP incorporated per min in a 20-min assay).

We also measured activity in an RNA polymerase assay that uses denatured salmon sperm DNA template (Hodo and Blatti, 1977). This assay revealed a 4.3-fold lower specific activity for rpb2–10 versus wild type pol II (0.4 versus 1.7 units/mg; 1 unit = 1 pmol CTP incorporated per min in a 20-min assay). Taken together, these three assays suggest that rpb2–10 pol II is generally 2–4-fold slower than the wild type in its average elongation rate.

Nascent RNA Cleavage by RPB2 Mutants—Because the mutant enzymes displayed a slowed response to SII in read-through of these arrest sites, we assayed the gross ability of each enzyme to cleave the nascent RNA in the arrested complex, an obligatory step in SII-dependent read-through (Reines, 1994). Arrested complexes were isolated with an anti-RNA antibody and washed free of nucleoside triphosphates prior to treatment with SII. Each mutant enzyme was capable of shortening the nascent transcript in an SII-dependent fash-

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**Fig. 7.** A, transcript elongation on pUC18 tailed template. Transcription reactions were performed using wild type (left panel) or rpb2–10 (right panel) pol II, and aliquots were removed and stopped at the indicated times. Position of run-off (RO) transcripts (approximately 2660 nucleotides) is indicated at right. Positions of RNA markers (1164, 540, 420, 380, and 260 nucleotides) are indicated by the arrowheads at left. B, percent of run-off transcripts versus time. Quantitation of data in A for wild type (solid triangles) and rpb2–10 (open triangles) pol II. % run-off = run-off/mean total radioactivity in lane. C, percent transcripts 980 nucleotides or larger versus time. Quantitation of data in A for wild type (solid triangles) and rpb2–10 (open triangles). % > 980 = RNAs > 980 nucleotides or larger/average amount radioactivity loaded per lane. The asterisk indicates value significantly different than wild type (p < 0.05 by Student’s t test; n = 3)
rested complexes were assembled as described in the legend for Fig. 3. Complexes arrested at site Ia were immunoprecipitated, washed free of NTPs and incubated for 30 min in the absence (lanes 1) or the presence (lanes 2 and 3) of SII (approximately 30-fold molar excess) and 800 μM of ATP, UTP, GTP, and CTP (lanes 3 only). Positions of arrested (la) and cleaved (*) transcripts are indicated. A–D show reactions employing wild type, rpb2–4, rpb2–7, and rpb2–10 enzymes, respectively. In E, arrested rpb2–10 complexes were washed free of NTPs (lane 1) and treated with SII and 800 μM of ATP, UTP, GTP, and 3′dCTP (lane 2).

Note: The symbol rpb2–10 in intrinsic arrest elements average elongation rate of the rpb2–10 cleavage product produced by the rpb2–10 A–D ion (Fig. 8, 9 lane 2 and Figs. 3, 4, and 6). RNA cleavage by the rpb2–10 enzyme could be shown more clearly by including a chain-terminating nucleotide, 3′dCTP, in the reaction. This allowed extension of the shortened products to a single, 3′ dCMP-terminated transcript upstream of the original arrest site, indicating that the nascent transcript had indeed been quantitatively cleaved to a shorter intermediate prior to re-extension (Fig. 8E).

DISCUSSION

In this study we have identified three mutant alleles of the second largest subunit of yeast pol II, rpb2–4, rpb2–7, and rpb2–10 (Scafe et al., 1990a, b), that confer sensitivity to growth of cells in the presence of 6AU and yield defective pol II enzymes. This is, to our knowledge, the first report of a 6AU-sensitive mutation in pol II that has been shown to yield an altered enzyme activity in vitro.

Presumably, this drug's effect on cellular NTP pools puts a stress on the elongation machinery, resulting in a growth defect (Archambault et al., 1992). This hypothesis seems particularly attractive considering the demonstrated relationship in vitro between arrest efficiency and dwell time (Wiest and Hawley, 1990; Wiest et al., 1992; Gu and Reines, 1995a). A similar relationship has been observed between the rate of nucleotide addition and termination for other RNA polymerases, including slow, termination-prone mutants of E. coli RNA polymerase (Jin and Gross, 1991) and RNA polymerase III (Shaaban et al., 1995). We would expect that depressing the ATP and CTP pools would manifest a growth defect in these and other elongation defective mutants.

These in vitro results are consistent with the in vivo findings relating sensitivity to growth on 6AU with compromised elongation efficiency. The 6AU-sensitive mutations rpb2–4 and rpb2–10 decrease the efficiency with which pol II reads through intrinsic arrest elements in vitro in the absence of SII. The average elongation rate of the rpb2–10 mutant is also reduced. We suggest that the arrest-prone phenotype conferred by this mutation is at least partially due to the slowed overall elongation rate of the mutant pol II molecule.

Although the extent of read-through in the presence of SII is relatively unaffected, the rate at which full-length transcript production is completed is reduced for the rpb2–10 enzyme relative to wild type. This might be expected for a number of reasons. First, more arrested pol II molecules are found at each arrest site for the mutant than the wild type enzyme, so the increased probability of arrest per encounter is likely to mean that on average, more rounds of cleavage and read-through are required for each pol II molecule (Reines, 1994). Second, the presence of tandem arrest sites means a mutant deficiency can manifest itself at, on average, more positions per template than wild type enzyme. Thus, a greater accumulation of enzymes at site II leads to decreased read-through at downstream arrest sites and a higher overall dependence on SII. Finally, the average rate of elongation between arrest sites and between the final arrest site and the end of the template is reduced relative to wild type. That SII enables an arrest-prone mutant pol II to achieve complete read-through is consistent with the rescue of 6AU sensitive pol II alleles by SII overexpression in vivo.

rpb2–10 (P1018S) and rpb2–2 (A1016T) are conditional mutations in homology region H of yeast pol II's second largest subunit (Scafe et al., 1990a, b). These residues are very highly conserved across many species, including representatives of archaeabacteria, eubacteria, fungi, higher plants, many invertebrates, and humans (Iwabe et al., 1991; Sidow and Thomas, 1994). Two conditional lethal alleles in the second largest subunit of Drosophila pol II, Z43 (R940H) and M39 (G982E), map in or very near region H (Chen et al., 1993). Mutation of Lys979 or Lys987 to Arg in homology region H of Saccharomyces cerevisiae RPB2 is lethal, and one or both of these residues can be cross-linked to derivatized nucleotides (Treich et al., 1992). Thus, genetic and biochemical evidence emphasizes the importance of region H in pol II function and suggests it is part of a catalytic pocket. If an alteration in this region affects nucleotide affinity or catalytic efficiency, slowed reaction speed (i.e. a longer dwell time at each template position) could result. Reduced reaction rate would mimic the effect of low substrate concentration, placing the enzyme at increased risk of becoming arrested when it encounters an arrest signal in DNA. This predicts that other slow pol II's, such as those bearing the α-amanitin-resistant C4 mutation in the largest subunit of Drosophila pol II (R741H; Coulter and Greenleaf, 1985), would also be arrest-prone.

It is important to note that in these in vitro assays, the read-through ability of mutant pol II enzymes did not correlate with the relative 6AU sensitivity of the strains bearing mutant alleles. rpb2–7 pol II reads through arrest sites with much greater efficiency than rpb2–10. However, strain Z425, which expresses rpb2–7 pol II, was the most sensitive to 6AU and mycophenolic acid, whereas strain Z428, bearing rpb2–10, exhibited a milder 6AU sensitivity. This suggests that the molecular basis for growth inhibition by 6AU is complex and may involve components outside the core enzyme or transcription activities not scored in a promoterless elongation assay.

The best characterized elongation factors that affect pol II arrest frequency are TFIIIF and SII. We can rule out the possibility that association of these pol II binding proteins varies between our preparations of rpb2–7 and wild type enzymes because the detergent sarcosyl, which inhibits both of these factors, does not alter the arrest frequencies we measure. As well, our assay system includes heparin to prevent reinitiation and therefore is insensitive to the presence of TFIIIF (Tan et al., 1995). Allele-specific extragenic suppression of rpb2–7 might identify other factors that differentially affect arrest frequency of rpb2–7 pol II to cause 6AU sensitivity in vivo.
pol II’s nucleotide binding region, polymerase active site, nuclease active site, and putative product groove may lie in very close proximity within the enzyme (Nudler et al., 1995; Wang et al., 1995). The polymerase and nuclease active sites have been suggested to be one and the same (Rudd et al., 1994). Therefore, it is possible that mutations in a single amino acid residue could cause perturbations affecting more than one biochemical function of the enzyme. Although it is parsimonious to postulate that reduced catalytic efficiency of pol II leads to increased arrest and a slowed response to SII, the rp2b-10 enzyme may have additional transcriptional defects. Apparent heterogeneity in the lengths of arrested transcripts, in contrast to the more discrete products produced by wild type pol II, suggests a heterogeneity in the recognition of initiation and/or arrest sites. However, primer extension mapping of the 5’ ends of RNA synthesized by these two enzymes revealed no difference in their start sites on tailed templates, although it confirmed the heterogeneity of initiation on tailed templates (Dedrick and Chamberlin, 1985). Arrest at site la by wild type enzyme is intrinsically heterogeneous with RNAs ending at three consecutive bases (Reines et al., 1987; Gu et al., 1993). Given the unusual heterogeneity of rp2b-10’s transcripts, it is difficult to determine nascent RNA cleavage rates and identify cleavage product sizes. Use of a promoter-based transcription system to test pol II activities of these mutants will be important to confirm the elongation defects, identify other pol II functions that may be impaired in 6AU-sensitive pol II, and extend the analysis of the cleavage reaction.

Acknowledgments—We acknowledge N. Hannett and R. A. Young for bringing to our attention the 6AU growth sensitivity of the strains described here and for communicating the unpublished nucleotide binding region of pol II’s, polymerase active site, and putative product groove. Acknowledgments—We acknowledge N. Hannett and R.A. Young for bringing to our attention the 6AU growth sensitivity of the strains described here and for communicating the unpublished nucleotide binding region of pol II’s, polymerase active site, and putative product groove.

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J. Biol. Chem. 1996, 271:6866-6873.
doi: 10.1074/jbc.271.12.6866

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