Intrinsic Transcript Cleavage in Yeast RNA Polymerase II Elongation Complexes*

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Rodney G. Weilbaecher‡, Donald E. Awrey%, Aled M. Edwards%, and Caroline M. Kane‡||
From the ‡Department of Molecular and Cell Biology, University of California, Berkeley, California 94720 and the ||Banting and Best Department of Medical Research, C. H. Best Institute, University of Toronto, Toronto, Ontario M5W 1L6, Canada

Transcript elongation can be interrupted by a variety of obstacles, including certain DNA sequences, DNA-binding proteins, chromatin, and DNA lesions. Bypass of many of these impediments is facilitated by elongation factor TFIIS through a mechanism that involves cleavage of the nascent transcript by the RNA polymerase II/TFIIS elongation complex. Highly purified yeast RNA polymerase II is able to perform transcript hydrolysis in the absence of TFIIS. The “intrinsic” cleavage activity is greatly stimulated at mildly basic pH and requires divalent cations. Both arrested and stalled complexes can carry out the intrinsic cleavage reaction, although not all stalled complexes are equally efficient at this reaction. Arrested complexes in which the nascent transcript was cleaved in the absence of TFIIS were reactivated to readthrough blocks to elongation. Thus, cleavage of the nascent transcript is sufficient for reactivating some arrested complexes. Small RNA products released following transcript cleavage in stalled ternary complexes differ depending upon whether the cleavage has been induced by TFIIS or has occurred in mildly alkaline conditions. In contrast, both intrinsic and TFIIS-induced small RNA cleavage products are very similar when produced from an arrested ternary complex. Although α-amanitin interferes with the transcript cleavage stimulated by TFIIS, it has little effect on the intrinsic cleavage reaction. A mutant RNA polymerase previously shown to be refractory to TFIIS-induced transcript cleavage is essentially identical to the wild type polymerase in all tested aspects of intrinsic cleavage.

Gene expression can be controlled by regulating any step of the transcription cycle: promoter binding, initiation, promoter escape, elongation, or termination. After transcript synthesis begins, the ability to suppress or complete the synthesis of an RNA transcript is vital to the cell, and a substantial and growing number of genes have been reported to be regulated during transcript elongation (1).

The ternary elongation complex consists of the RNA polymerase, the template DNA, and the nascent RNA transcript. Surratt et al. (2) discovered that bacterial RNA polymerase II ternary complexes have a mechanism for transcript shortening, endonucleolytic cleavage near the 3'-end of the transcript. After transcript cleavage, the 3'-fragment is released while the 5'-fragment is retained in an active ternary complex. A single cleavage event can liberate from 1 to 17 nt of RNA bearing a 5'-monophosphate (3–6). Remarkably, transcription accurately resumes from the site of transcript cleavage to re-synthesize the excised RNA.

Transcript cleavage activity is conserved in many DNA-dependent RNA polymerases, including bacterial RNA polymerases, vaccinia virus RNA polymerase (7), RNA polymerase I (8, 9), RNA polymerase II (10–14), and RNA polymerase III (15). Accessory factors that stimulate transcript cleavage in ternary complexes have been identified in both prokaryotes (GreA and GreB) and eukaryotes (reviewed in Refs. 3, 9, 16–18). A subunit of vaccinia virus RNA polymerase, rpo30, is 25% similar to one such elongation factor, TFIIS (19), and this vaccinia subunit is thought to affect the transcript cleavage reaction of the vaccinia polymerase.

Several observations suggest that the catalytic site for this transcript hydrolysis resides within the polymerase. First, RNA polymerase III complexes catalyze transcript cleavage, but a separate cleavage-stimulating factor has not been reported for RNA polymerase III. Second, ternary complexes of Escherichia coli RNA polymerase purified from a greA− greB− strain retain a low level of transcript cleavage activity (20), an activity stimulated by basic pH. Third, mammalian RNA polymerase II pyrophosphorylates its transcript to give products identical to those induced by treatment with TFIIS (21). However, how TFIIS or the prokaryotic Gre factors stimulate this intrinsic cleavage remains unknown.

For yeast RNA polymerase II (RPII), the Rpb9p subunit has been shown to mediate the signal between TFIIS and the polymerase catalytic center for stimulating transcript cleavage (22). Arrested ternary complexes formed with yeast RNA polymerase II lacking Rpb9 (RPIIΔ9) are much less responsive to TFIIS, and yet they can carry out the intrinsic cleavage reaction. These results led us to characterize the transcript cleavage reaction of RPII in more detail, particularly to compare the intrinsic cleavage in stalled and arrested complexes. Furthermore, we compared the wild type and the RPIIΔ9 polymers in their intrinsic cleavage properties. The analysis of intrinsic transcript cleavage activity in several discrete elongation complexes suggests extensive structural and/or functional hetero-

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‡Present address: Dept. of Molecular and Cellular Oncology, University of Texas, M. D. Anderson Cancer Center, Box 108, Rm. Y8.6073, 1515 Holcombe Blvd., Houston, TX 77030.

§To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley, CA 94720. Tel.: 510-642-4118; Fax: 510-642-5227; E-mail: kanecm@ uclink4.berkeley.edu.

The abbreviations used are: nt, nucleotide(s); RPII, RNA polymerase II; CAPS, 3-(cyclohexylamino)1-propanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

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genity during transcription elongation. The results provide insights for how TFIIS might influence the properties of an elongation complex, aside from accelerating transcript cleavage.

**EXPERIMENTAL PROCEDURES**

**Reagents**

High performance liquid chromatography-grade NTPs were purchased from Amersham Biosciences. [α-32P]CTP and [α-32P]UTP were purchased from PerkinElmer Life Sciences. Disodium pyrophosphate, α-amanitin, and CAPS were purchased from Sigma. Streptavidin-coated magnetic beads (Dynal Beads) were purchased from Dynal. Biotin-14-dCTP was purchased from Invitrogen. Terminal deoxynucleotidyl transferase was purchased from either Invitrogen or Roche Biochemicals (Los Alamos, NM). Biospin-30 columns were purchased from Bio-Rad.

**Purification of RNA Polymerase II and TFIIS**

DNA polymerase II (RPⅡ) lacking Relp9 (RPⅡR9) was purified from the Saccharomyces cerevisiae strain W303 (27). Both RPⅡ and RPⅡR9 were purified as described previously (24, 25), except that 10 μM ZnCl2 was included in all buffers. The polymerase preparations were stored at −80°C in 10 mM Tris-HCl, pH 7.9, 40 mM ammonium sulfate, 10% glycerol, 2.5 mM DTT, 100 mM EDTA, and 10 μM ZnCl2. Recombinant yeast TFIIS was purified as previously described (25).

**Plasmids and DNA Templates**

Plasmid pRW102 was constructed by digesting pGR220 (26) isolated on a SacI-KpnI fragment into the same sites of pTK242A (28) in which 17 bp were deleted by digesting with Sau3AI sites. The pRW102 plasmid was digested completely with SmaI sites. The pRW102 plasmid was digested with SmaI and re-circularized, generating pRW104 with a single Smal site. Plasmid pRW106 was constructed by digesting pGWI104 with Nsil, treating with T4 DNA polymerase to trim the overhangs, then digesting with HindIII to isolate the T1A-containing insert. This segment was inserted into the HindIII and HincII sites of pN2174. pN2174 is a derivative of pN2 (28) in which 17 bp were deleted by digesting with EcoRI and BamHI, treating these ends with Klenow, and finally re-ligation.

Transcription templates from pRW106 were generated by digestion with SmaI, then 3'-deoxycytidylate extensions were added with terminal nucleotidyl transferase (29). Templates constructed with such 3' tails are referred to as pCpRW106 templates. For 3'-biotinylated templates, unincorporated dCTP was removed by using two consecutive Biospin-30 columns (blocked with 20 μg/ml RNAse), followed by incoporation of biotin-14-dCTP with terminal nucleotidyl transferase. Unincorporated biotin-14-dCTP was removed by centrifugal gel filtration. Finally, pCpRW106 templates were restricted with NciI and EcoRI to generate a run-off transcript and to disable transcription from the undesired end of the template. The pGCpGEMTERM template preparation was described previously (14); this template harbors a series of arrest sites in the first intron of the human histone H3.3 gene (30).

**Buffers and Denaturing Gels**

All transcription reactions were initiated using standard transcription buffer (70 mM Tris-OAc, pH 8.0, 60 mM Na2OAc, 5 mM MgOAc, 5% glycerol, 1 mM DTT). Alkaline intrinsic transcript cleavage buffer contained 70 mM CAPS, pH 9.5, 100 mM KCl, 5 mM MgCl2, 5% glycerol, 1 mM DTT. Manganese intrinsic transcript cleavage buffer contained 70 mM Tris-Cl, pH 8.0, 100 mM KCl, 5 mM MnCl2, 5% glycerol, 1 mM DTT. Control transcript cleavage buffer contained 70 mM Tris-Cl, pH 8.0, 100 mM KCl, 5 mM MgCl2, 5% glycerol, 1 mM DTT, 2 mM SDS/urea stop mix contained 10 mM urea, 0.5% SDS, 90 mM Tris borate, pH 8.0, 10 mM EDTA, 0.05% bromphenol blue, and xylene cyanol. 1× TBE gel buffer contained 90 mM Tris borate, pH 8.0, and 25 mM EDTA.

The 135, 138, 143, 152, and 158 nt transcripts formed on pCpRW106 were resolved by gel electrophoresis in 7.5% polyacrylamide (38% acrylamide:2% bisacrylamide) gels containing 7 μM urea in 1× TBE. The T1A (~200 nt), C1, and C2 transcripts (25) formed on pCpGEMTERM were resolved on 6% polyacrylamide (38% acrylamide:2% bisacrylamide) gels containing 7 μM urea in 1× TBE. RNA oligomers released during transcript cleavage were resolved on either 15% (38% acrylamide:2% bisacrylamide) or 20% (40% acrylamide:6% bisacrylamide) gels containing 7 μM urea in 1× TBE.

**Imobilized DNA Templates**

Biotinylated DNA templates (prepared as described above) were coupled to streptavidin-coated magnetic Dynabeads prior to transcription.

**Formation of Ternary Complexes on Immobilized Templates**

G135 ternary complexes were formed by incubating 1 pmol of RNA polymerase II with 5 pmol of Dynabead-conjugated pCpRW106 in 1× transcription buffer containing 1 μM [α-32P]CTP (3000 Ci/mmol), 800 μM GTP and ATP, and 1 unit of InhibitAce, in a final volume of 25 μl for 1.5 min at 30°C. At this time, ternary complexes containing short transcripts (~13 nucleotides) predominate (data not shown). After this 5'-end-labeling step, 175 μl of 1× yeast transcription buffer containing 100 μM CTP and 0.1 mg/ml heparin was added, and incubation continued for 1.5 min at 30°C. Acetylated BSA at 25 μg/ml was included in all transcription buffers to inhibit the nonspecific adsorption of streptavidin to the microcentrifuge tubes. Unincorporated nucleotides were removed by collecting the ternary complexes via magnetic precipitation and washing three times with 50 μl of transcription buffer.

G135 ternary complexes were “walked” to form U138 ternary complexes by addition of transcription buffer containing 10 μM UTP followed by incubation for 3 min at 30°C. U138 ternary complexes were purified from unincorporated UTP by magnetic precipitation, and two washes with 50 μl of transcription buffer. U138 ternary complexes were walked to form G143 ternary complexes by addition of transcription buffer containing 10 μM CT and GTP followed by incubation for 3 min at 30°C. G143 ternary complexes were formed by incubation for 3 min at 30°C. G152 ternary complexes were purified from unincorporated NTTPs by magnetic precipitation and two washes with 50 μl of transcription buffer. These were walked to C158 upon addition of 10 μM CTP and ATP for 10 min at 30°C.

Ternary complexes halted on pCpGEMTERM, containing the T1A block to elongation (31), were formed by incubating 1 pmol of RNA polymerase II with 5 pmol of Dynabead-conjugated pCpGEMTERM in 1× transcription buffer containing 1 unit of InhibitAce, 1 μM [α-32P]CTP (3000 Ci/mmol), 800 μM UTP, GTP, and ATP, in a volume of 25 μl for 1.5 min at 30°C. After the addition of CTP to 100 μM, heparin to 0.1 mg/ml, and BSA-OAc to 25 μg/ml, reactions were incubated for 1.5 min at 30°C to generate ternary complexes halted at T1A, T1B, and T1I. Ternary complexes were purified from unincorporated NTTPs by magnetic precipitation and three washes with 50 μl of transcription buffer.

3'-End Labeling of U138 and T1A Ternary Complexes

3'-End-labeled U138 Complexes—Unlabeled G135 complexes were formed on immobilized pCpRW106 as described above with the exception that unlabeled CTP was substituted for [α-32P]CTP. The unlabeled G135 complexes were purified from unincorporated NTTPs by magnetic precipitation and washed three times with 50 μl of 1× standard transcription buffer. 3'-End-labeled U138 complexes were generated by addition of 3 μM [α-32P]UTP for 5 min at 30°C. Unincorporated nucleotides were removed as above.

3'-End-labeled T1A Complexes—Unlabeled T1A complexes were formed on immobilized pCpGEMTERM as described above with the exception that unlabeled CTP was substituted for [α-32P]CTP. The unlabeled G135 complexes were purified from unincorporated NTTPs by magnetic precipitation and three washes with 50 μl of 1× standard transcription buffer. 3'-End-labeled T1A complexes were generated by addition of 3 μM [α-32P]UTP in 1× standard transcription buffer and incubation for 5 min at 30°C. Unincorporated nucleotides were removed as above.

**Transcript Shortening Reactions**

Ternary complexes were purified from unincorporated NTTPs either by centrifugal gel filtration with Biospin-30 columns or through use of...
Transcript shortening was performed in 70 mM CAPS, KCl (or 100 mM NH4OAc), MgCl2 (or MnCl2), 5% glycerol, and 1 mM DTT. Alkaline-stimulated magnetic precipitation was used to isolate the ternary complexes (Fig. 1). However, all transcript-shortening reactions were more efficient when magnetic precipitation, as indicated in the figure legends. The method of ternary complex purification did not qualitatively influence results. The template was pCpGEMTERM (14). A minimal sequence sufficient for cleavage was the human histone H3.3 gene are presented. The template was pCpGEMTERM (14). A minimal sequence sufficient to block RNA polymerase II elongation complexes is boxed (27). C1 demarcates the position of yeast and mammalian ternary complexes after the initial transcript cleavage event from the T1a site (25, 37).

RESULTS

Transcript Cleavage in Arrested Complexes—Purified RNA polymerase II stops and arrests in vitro at several sites within the human histone H3.3 first intron (T1a, T1b, and T1I (30)). The strongest block, T1a, has frequently been used to study the elongation properties of RNA polymerase II from yeast and mammalian cells (27, 31–34). Approximately 50% of yeast or mammalian RNA polymerase II elongation complexes stop within the first tract of nontemplate thymidines at the T1a site (14, 27, 35) (Fig. 1A) and do not efficiently resume elongation in the absence of elongation factor TFIIS. Thus, they are "arrested" (16, 36) at the T1a site. RNA polymerase II arrested at T1a requires a transcript cleavage event, stimulated by TFIIS, to promote readthrough of the elongation block (37). Thus the T1a site has been used to investigate the mechanism of transcription arrest as well as the relief of arrest in the presence of TFIIS.

Biochemical Properties of the Intrinsic Cleavage Reaction—The addition of TFIIS to yeast RNA polymerase II elongation complexes arrested at T1a in the absence of NTPs generates a characteristic pattern of shortened transcripts (Fig. 2, lane 7) (see also Ref. 25). Two major cleavage products, C1 and C2, remain associated with active ternary complexes; the C1 cleavage product is generated prior to C2 (25). A low but detectable level of these cleaved transcripts was observed in the absence of TFIIS (Fig. 2, lane 2). This transcript cleavage intrinsic to RNA polymerase II was accelerated in mildly alkaline solutions (Fig. 2, lanes 3 and 4), as has been seen with E. coli RNA polymerase (20). Like the TFIIS-stimulated transcript cleavage, this intrinsic transcript cleavage yielded shortened transcripts associated with active ternary complexes that resumed elongation upon the addition of NTPs (Fig. 2, lane 5).

Intrinsic Cleavage Requires Metal Cofactors—The intrinsic cleavage reaction was dependent on the addition of divalent cation (Fig. 3). A survey of divalent cations revealed that 0.5 mM Mn2+ or Ca2+ (Fig. 3, lanes 3 and 4) facilitated substantially higher levels of intrinsic transcript cleavage than did Mg2+ (Fig. 3, lane 2) at pH 8. Concomitant addition of 5 mM MgCl2 did not reduce the level of intrinsic transcript cleavage supported by 0.5 mM MnCl2 or CoCl2 (Fig. 3, lanes 6 and 8). Shortened transcripts were elongated upon the addition of NTPs and thus were associated with active ternary complexes (Fig. 3, lanes 7 and 9). Other divalent cations such as Zn2+ and Cd2+ did not support TFIIS-stimulated transcript cleavage or intrinsic transcript cleavage at pH 8 (data not shown).

The effects of monovalent anions (Cl− and OAc−) and cations (K+, Na+, and NH4+) on the rate of intrinsic transcript cleavage were also examined. In contrast to the pronounced stimulation of transcript cleavage by mild alkaline or certain divalent cations, the overall effects of salts (100 mM) were generally subtle and did not resolve into cation-specific or anion-specific effects. However, 100 mM KCl was optimal for intrinsic transcript cleavage at pH 9.5 or with manganese at pH 8.0. Curiously, 100 mM NH4OAc was least effective in supporting intrinsic transcript cleavage (data not shown), despite the fact that ammonium is capable of stimulating the overall elongation reaction of RNA polymerase II (38, 39). Apparently, the stimulation of elongation by ammonium is not mediated by an increase in transcript cleavage at sites blocking elongation by RNA polymerase.

The Effect of NTPs and Pyrophosphate on the Intrinsic Cleavage Reaction—The addition of NTPs stimulates the transcript cleavage activity of vaccinia virus RNA polymerase (7) and...
The ternary complexes formed on the pCpGEMTERM template were purified and resuspended in transcription buffer lacking NTPs, Mg2+, and DTT. The starting material is depicted in lane 1. The ternary complexes were placed into reactions containing MgCl2, MnCl2, or/and CoCl2 at the indicated concentrations for 10 min at 30 °C. After this incubation, NTPs were added (0.8 mM each) to the reactions in lanes 6 and 8. Incubation was continued for 10 min at 30 °C, and the products were resolved in lanes 7 and 9, respectively.

RNA polymerase III (15). Thus, we also tested whether the intrinsic cleavage reaction of yeast RNA polymerase II was stimulated by the addition of NTPs. In contrast to the results with the viral polymerase and RNA polymerase III, NTPs did not stimulate the intrinsic transcript cleavage activity (data not shown) of two different yeast RNA polymerase II elongation complexes halted at either T1a or U138 (Fig. 1B, and see below). The TFIIIS-stimulated transcript cleavage by RNA polymerase II ternary complexes also is not stimulated by NTPs (data not shown and Ref. 13).

Pyrophosphate stimulates an endolytic event in an arrested complex containing mammalian RNA polymerase II (21). When yeast RNA polymerase II complexes arrested at the T1a site were treated with 2 mM pyrophosphate at pH 8, only a small number of shortened transcripts with mobility similar to C1 were observed (data not shown). Significantly higher levels of shortened transcript accumulated when the reaction was performed at pH 7 (Fig. 4, lane 1). The lack of stimulation under basic conditions is more consistent with a pyrophosphorolytic than a hydrolytic mechanism. Pyrophosphate-shortened transcripts were associated with active ternary complexes as evidenced by their ability to resume elongation upon addition of NTPs (Fig. 4, lane 2). However, these results suggest that pyrophosphorolysis does not account for the observed intrinsic cleavage activity.

**Fig. 3. Divalent cation requirements for intrinsic cleavage.** The ternary complexes formed on the pCpGEMTERM template were purified and resuspended in transcription buffer lacking NTPs, Mg2+, and DTT. The starting material is depicted in lane 1. The ternary complexes were placed into reactions containing MgCl2, MnCl2, or/and CoCl2 at the indicated concentrations for 10 min at 30 °C. After this incubation, NTPs were added (0.8 mM each) to the reactions in lanes 6 and 8. Incubation was continued for 10 min at 30 °C, and the products were resolved in lanes 7 and 9, respectively.

**Fig. 4. Pyrophosphorolysis in T1a elongation complexes.** Ternary complexes were formed and purified as in Fig. 1. Complexes were suspended in standard transcription buffer containing 2 mM sodium pyrophosphate at pH 7 and were incubated for 40 min at 30 °C (lane 1) followed by addition of NTPs (0.8 mM each) and continued incubation for 10 min at 30 °C (lane 2).

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Intrinsic Transcript Cleavage in Complexes Stalled by Withholding Nucleotides—Elongation complexes stopped at T1a represent naturally arrested ternary complexes that inefficiently resume elongation in the presence of optimal NTPs; the TFIIIS cofactor is essential to rescue the complexes from arrest at physiological pH. To examine whether intrinsic transcript cleavage occurs in other types of elongation complexes, five discrete complexes were examined that had been artificially halted by NTP omission. These complexes were prepared using a “walking” proto-
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| Cleavage Conditions | Control pH 8, Mg²⁺ | Intrinsically cleaved pH 9.5, Mg²⁺ | Intrinsically cleaved pH 8, Mn²⁺ | TFIIIS (1:50) pH 8, Mg²⁺ |
|---------------------|-------------------|----------------------------------|----------------------------------|------------------------|
| I. Cleavage (min)   | 20                | 20                               | 20                               | 20                     |
| II. Chase (min)     | 10                | 10                               | 10                               | 10                     |

Fig. 5. Transcript cleavage and readthrough by T1a-arrested ternary complexes treated with alkali, manganese, or TFIIIS. Purified ternary elongation complexes formed on pCpGEMTERM were incubated for 20 min at 30 °C as indicated in either standard transcription buffer (lane 1), alkaline buffer (lane 3), manganese-containing buffer (lane 5), or TFIIIS-containing buffer (lane 7; TFIIIS at a 1:50 molar ratio to RNA polymerase II). An aliquot was taken from each reaction, washed twice with transcription buffer, and then supplemented with 800 μM GTP and UTP, 100 μM CTP, and incubated at 30 °C for 10 min (lanes 2, 4, 6, and 8, respectively). This NTP subset allows C1 (asterisk) and T1a ternary complexes to elongate past the T1a site to generate a specific readthrough product (RT).

Fig. 6. Intrinsically cleaved RNAs in elongation complexes formed by withholding nucleotides. A, lanes 1 and 8 contain purified G135 and U138 complexes formed from the pCPpWR106 template (see Fig. 1B). Each complex was suspended as indicated in either standard transcription buffer (lanes 3 and 10), alkaline buffer (lanes 4 and 11), TFIIIS-containing buffer (lanes 6 and 13; TFIIIS at a 1:10 molar ratio to RNA polymerase II), or 1 molar pyrophosphate (PPi) containing buffer (lanes 7 and 14) for the indicated times at 30 °C. B, ternary complexes were walked from U138 to template positions G143, G152, or C158 as described under “Experimental Procedures,” and the complexes were purified. Aliquots from each ternary complex were resuspended in either standard transcription buffer at pH 8 (lanes 1, 3, and 5), or alkaline buffer at pH 9.5 (lanes 2, 4, and 6) and incubated for 20 min at 30 °C.

Intrinsic transcript cleavage was also observed with G135 and C158 complexes (Fig. 6B, lanes 4 and 6) but not in G143 elongation complexes in the timeframe of the experiment (Fig. 6B, lane 2, and data not shown). All of these complexes were active, and their transcripts could be elongated upon addition of NTPs (data not shown). Hence, over the course of 23 bp of template sequence, only three of five elongation complexes exhibited detectable intrinsic transcript cleavage during the experiment, whereas all complexes remain competent for elongation upon addition of NTPs. The reason that the other two stable complexes were refractory to detectable intrinsic cleavage is not known. Of the five ternary complexes had arrested, and yet the catalytic center of the enzyme apparently had greater mobility to move away from the 3′-end of the transcript in some but not other active complexes. Both G135 and G143 are G:C-rich upstream of the stalled position. The complex most susceptible to intrinsic cleavage, U138, has three Us at the 3′-end of the transcript. It has been proposed that a more mobile catalytic center, and thus more cleavage, might be seen over a A:T-rich sequences (46). Indeed, both G152 and C158 complexes undergo intrinsic cleavage to a more limited degree than U138, and those two complexes have terminal G or C residues.

Similarities and Differences in the Sites of Intrinsic and TFIIIS-stimulated Transcript Cleavage—If the primary role of TFIIIS were to stimulate the intrinsic RNA polymerase endonuclease activity, then one would expect that the pattern, but not necessarily the kinetics, of cleavage would be identical in the presence or absence of TFIIIS. To test this hypothesis, we compared the cleavage patterns produced by the intrinsic reaction and by TFIIIS in the T1a and U138 elongation complexes. In 3′-end-labeled U138 complexes, mild alkaline (pH 9.5) or manganese stimulated the release of a predominant RNA product with a mobility consistent with a trimer, although the exact size is not known. However, it is clear that intrinsic cleavage generates a set of products distinct from those released follow-
ing stimulation by TFIIH (Fig. 7A, bottom, compare lanes 4 and 6 with lane 9). That is, TFIIH stimulated the release of three products, none of which migrated with the mobility of the product detected from the intrinsic cleavage reaction (Fig. 7A, bottom, lane 9). When TFIIH was incubated with the U138 complexes under the intrinsic cleavage stimulating conditions, oligomers were the same size as those released by TFIIH-stimulated cleavage under standard conditions (Fig. 7A, bottom, lane 10, and data not shown). Thus, the differences in cleavage products could not be explained simply by differences in the reaction conditions. Furthermore, the efficiency of cleavage induced by TFIIH was greater than that induced by basic pH. These results strongly suggest that TFIIH influences the site of transcript hydrolysis, in addition to stimulating the rate of transcript hydrolysis.

Complexes containing 3′-end-labeled T1a transcripts were also examined for the sizes of liberated cleavage products (Fig. 7B, bottom). The analyses of the T1a-derived transcripts are complicated somewhat because of the presence of minor contaminating transcripts, but two general features are evident. First, many of the intrinsic and TFIIH-stimulated cleavage products from the 3′-end-labeled arrested T1a complexes are of the same mobility (compare lanes 3, 4, and 6 in “released products”), which is in contrast to the disparity in sizes of...
released cleavage products from the stalled U138 complex. Second, pyrophosphate-treated 3'-labeled T1a complexes also liberate two products with the same low mobility as seen with TFIIS or intrinsic cleavage (Fig. 7B, bottom, lane 7). The behavior of the U138 and T1a elongation complexes is clearly distinct for transcript cleavage. Thus, the intrinsic cleavage reaction is affected by both the site of the elongation block as well as the method used to create the block.

*α*-Amanitin Inhibits TFIIS-stimulated Cleavage but Not Intrinsic Cleavage—The fungal toxin α-amantin is a well known inhibitor of RNA polymerase II transcription. The toxin binds tightly to RNA polymerase II (47), blocks TFIIS-stimulated transcript cleavage for some complexes (14, 48), and significantly slows elongation and pyrophosphorolysis (49). Given these effects, α-amantin might also interfere with the intrinsic transcript cleavage reaction. Both the T1a and U138 ternary complexes were tested. Reactions with 150 μg/ml α-amantin effectively blocked TFIIS-stimulated transcript cleavage in both the T1a complex (Fig. 8, A and B, lanes 3–5) and U138 complex (Fig. 8, C and D, lane 6). Surprisingly, intrinsic transcript cleavage, stimulated by either mild alkaline or manganese at pH 8, was not inhibited by 150 μg/ml α-amantin in either the T1a (Fig. 8, A and B, lane 2) or U138 complexes (Fig. 8, C and D, lanes 4 and 5). For the T1a complex reconstituted in 5 mM manganese, 23 and 25% intrinsic cleavage was observed in the absence and presence, respectively, of 150 μg/ml α-amantin. For the alkaline-treated U138 complex, 35 and 25% intrinsic cleavage was observed in the absence and presence, respectively, of 150 μg/ml α-amantin. These results demonstrate that α-amantin does not directly inhibit the intrinsic hydrolytic activity of RNA polymerase II. Because the toxin does inhibit TFIIS-induced cleavage, it must interfere with some step specific to TFIIS in the stimulation of transcript hydrolysis. Alternatively, these conditions of mild alkali or manganese, pH 8, might interfere with the binding of amantin to the polymerase. However, this explanation does not hold for Mn²⁺, because amanitin has been shown to inhibit transcription *in vitro* in the presence of manganese (data not shown).

An RNA Polymerase II Mutant with Altered TFIIS Responsiveness Has Wild Type Intrinsic Cleavage Properties—Work with mutants of TFIIS suggested that the reactivation of arrested complexes requires at least two steps that are stimulated by TFIIS: cleavage of the nascent transcript and a hypothetical conformational change within the complex (35, 25). A mutant yeast RNA polymerase II lacking subunit Rpb9 (RPB19) is significantly impaired in its ability to respond to TFIIS-stimulated readthrough of elongation blocks, although the mutant polymerase binds TFIIS as well as wild type polymerase (25). It seemed possible that the molecular defect of RPII9 in response to TFIIS was in the intrinsic cleavage activity. However, complexes formed at U138 or T1a with RPII9 had no detectable reduction in intrinsic transcript cleavage relative to the wild type enzyme (data not shown).

Mild alkaline (pH 9.5) or manganese at pH 8 liberated the same intrinsic cleavage products at about the same rate and extent as with the wild type enzyme (Table I). Also, for both U138 and T1a complexes the intrinsic cleavage reaction was sufficient to promote readthrough with RPII9 (Table I provides data with T1a complexes). Thus, there appears to be no inherent defect in the intrinsic hydrolytic activity in RPII9. The impaired ability of this mutant polymerase to respond to TFIIS cannot be easily explained by these results. Further work using TFIIS mutants in combination with RPII9 should be informative.

Previous studies demonstrated that a mutant RNA polymerase II lacking both the RPB4- and RPB7-encoded subunits is similar to wild type enzyme in the rate of transcript elongation, comparison.

**Fig. 8.** Effect of α-amantin on intrinsic cleavage. A and B, T1a complexes. Purified ternary complexes were incubated without divalent cations (–Me²⁺) for 5 min at room temperature in the absence (A) or presence (B) of 150 μg/ml α-amantin. The complexes were then incubated an additional 15 min at 30 °C without further addition (lanes 1), or after the addition of 5 mM MnCl₂ (lanes 2), or 5 mM MgCl₂ and TFIIS (TFIIS at 5:1, 0:5:1, 0:05:1 ratio to RNA polymerase II in lanes 3-5 respectively). RNAs were resolved on a 6% denaturing gel. Asterisks denote cleavage products. C and D, U138 complexes. Purified U138 complexes were incubated without divalent cations (–Me²⁺) for 5 min at room temperature in the absence (C) or presence (D) of 150 μg/ml α-amantin. The complexes (lanes 2) were then incubated an additional 15 min at 30 °C in standard transcription buffer (lanes 3), alkaline buffer (lanes 4), manganese-containing buffer (lanes 5), or standard transcription buffer containing TFIIS (5:1 molar ratio to RNA polymerase II). Transcripts from the G135 complexes are included for size comparison.
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Table I

Quantitation of transcript cleavage and readthrough with RPH and RPH9 in T1a complexes in response to TFII S, mild alkali, and manganese

The experiments were done as outlined in Fig. 5 using either wild type RNA polymerase II or RNA polymerase II lacking Rpb9p. Percent transcript cleavage was calculated as (C1)/(C1) + (T1a). Percent readthrough was calculated as (RT)/(RT) + (T1a). (C1), (T1a), and (RT) are as defined in the legend to Fig. 5. These data are from a single experiment representative of several experiments carried out under different conditions and times.

|                | RPH                  | RPH9                 |
|----------------|----------------------|----------------------|
|                | Cleavage             | Readthrough          | Cleavage             | Readthrough          |
| Control (pH 8, Mg\(^{2+}\)) | 1.0                  | 16                   | 5.0                  | 17                   |
| TFII S (pH 8, Mg\(^{2+}\)) | 33                   | 30                   | 16                   | 19                   |
| Intrinsic (pH 9.5, Mg\(^{2+}\)) | 68                   | 42                   | 85                   | 63                   |
| Intrinsic (pH 8, Mn\(^{2+}\)) | 41                   | 29                   | 38                   | 28                   |

the recognition of intrinsic blocks to elongation, and the response to TFII S-stimulated cleavage and readthrough (14, 50). The intrinsic cleavage properties of this enzyme were tested with the U138 complex, and both the intrinsic and the TFII S-stimulated cleavage products and rates were indistinguishable from that of the wild type enzyme (data not shown). Thus, RPB4, RPB7, and RPB9 are dispensable for intrinsic transcript cleavage by RNA polymerase II.

Discussion

This work was carried out to explore the mechanisms of pausing, arrest, and readthrough by RNA polymerase II. The protein factor, TFII S, promotes readthrough of RNA polymerase II ternary elongation complexes that have arrested in vitro. This factor stimulates transcriptional cleavage at the 3’-end of the nascent transcript as a prerequisite for readthrough. The presented work focused on the factor-independent, intrinsic ability of RNA polymerase II to carry out this transcript cleavage reaction in both arrested and stalled complexes. Comparing intrinsic and TFII S-induced cleavage in these two types of complexes has identified both similarities and differences that highlight what parts of the readthrough reaction are ripe for regulation.

The transcript cleavage reaction has been conserved among all tested RNA polymerases and is important in the control of RNA elongation (16). For RNA polymerase II, a fraction of ternary complexes cease elongation within the transcription unit in a regulated fashion (1). In vivo, it is experimentally difficult to determine if these complexes have paused or have arrested. In vitro, arrested complexes appear to require transcript cleavage as a prerequisite for the resumption of elongation (13, 37, 51), and, at physiological pH, stimulation of cleavage requires TFII S (3, 52). However, by altering the biochemical conditions, intrinsic cleavage by the polymerase can be accelerated in arrested or stalled complexes, and this cleavage can be sufficient for promoting continued elongation.

In contrast to arrested complexes, interruption of transcript elongation by limiting subsets of NTPs often results in stable, paused elongation complexes that readily resume elongation upon addition of NTPs (Ref. 4, although see Ref. 53). These active ternary complexes often will arrest if incubated for an extended period in the absence of the next nucleotide needed for elongation (54–56). However, only a subset of the complexes stalled by depriving them of nucleotides, detectably carries out the intrinsic cleavage reaction. At the least, these observations indicate that stable, paused elongation complexes differ kinetically in the intrinsic cleavage reaction, and it remains possible that some complexes simply will not carry out this reaction. These results raise several questions. How do arrested complexes differ from stalled elongation complexes in their susceptibility to intrinsic cleavage? Why do different stalled elongation complexes differ in their susceptibility to intrinsic cleavage? Transcending these mechanistic questions is the regulatory dilemma: how can the cleavage reaction intrinsic to RNA polymerase II be stimulated by protein factors such as TFII S?

There are several models proposed to explain such functional differences (4, 46, 57–59). All of the models suggest that hydrolytic cleavage of the nascent transcript within an arrested or stalled ternary complex generates a new transcript 3’-end at the active site. As hypothesized, the cleavage reaction becomes necessary as the catalytic center of the polymerase is proposed to be mobile and can move away from the 3’-OH necessary for continued catalysis. This mobility can be in a readily reversible equilibrium, or it can result in a catalytic center arrested away from the transcript 3’-end. Transcript cleavage generates a new 3’-end, immediately proximal to the catalytic center, which permits continued incorporation of nucleotide substrates for catalytic elongation of the transcript.

A focus on the intrinsic cleavage reaction in this work removed one additional level of biochemical complexity, the stimulation of this reaction by protein factors. However, the comparison between the reaction intrinsic to the polymerase and that accelerated by TFII S was essential to evaluate what was carried out solely by the polymerase versus what was affected by the regulatory protein, TFII S.

As would be anticipated for a hydrolysis reaction, mild alkali stimulated intrinsic cleavage in several yeast RNA polymerase II ternary complexes. However, the present analysis does not distinguish between general base catalysis and specific base catalysis stimulated by elevated pH. Specific base catalysis has been proposed for the 3’→5’-exonuclease (proofreading) active site of the Klenow fragment, in which the two divalent metal ions in the exonuclease active site of DNA polymerase I apparently promote rapid hydrolytic cleavage of the phosphodiester bond (60). In contrast to specific base-catalyzed reactions, if an active site functional group were participating in catalysis as a general base, the ratio of the reaction should become independent of pH after the pK\(_a\) of the functional group is reached (61). For ternary transcription complexes, intrinsic transcript cleavage was significantly stimulated over the range of pH 8.0–9.5, but further elevation in pH (10.5) was only slightly more effective than pH 9.5 for intrinsic cleavage. However, at pH 10.5 a significant proportion (50%) of RNA polymerase II ternary complexes were inactivated for subsequent elongation (data not shown). A similar pH plateau has been observed for intrinsic cleavage with E. coli RNA polymerase ternary complexes (20), and these authors proposed that titration of a specific amino acid (i.e. histidine, lysine, and cysteine) is more likely than general (passive) base catalysis. It is not possible to distinguish between general and specific base-catalyzed hydrolysis reactions for the yeast RNA polymerase II, because the polymerase is inactivated in strong alkaline solutions, and there is differential stimulation of cleavage by some divalent cations.

The conditions identified here that stimulate intrinsic tran-
transcript cleavage are consistent with the prior work leading to the proposal that transcript cleavage is mediated at the active site of RNA polymerase. Pyrophosphorolysis can liberate large RNA oligomers from complexes stopped at natural blocks to elongation (this work) or by nucleotide exclusion (21). Furthermore, the mobilities of the RNA products that result from transcript cleavage within a single arrested ternary complex are similar whether cleavage is mediated intrinsically, by TFIIS, or by pyrophosphate. The same subset of divalent metal cofactors that support (Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$) and inhibit (Ca$^{2+}$ and Zn$^{2+}$) the TFIIS-mediated transcript cleavage reaction also support and inhibit the intrinsic reaction. This observation supports the notion that TFIIS, which accelerates the elongation (this work) or by nucleotide exclusion (21). Furthermore, implication of the cleavage reaction does not require a ternary complex that can operate directly on the polymerase when bound to RNA. Alternatively, it may be that TFIIS-mediated cleavage is influenced by RNA sequence (4), and certain RNA sequence contexts may have greater, or lesser, lability to hydrolysis. In this regard, it is noteworthy that the pattern of DNase I protection conferred by the U138 complex with mammalian RNA polymerase II is strikingly different from that of G135 and G143 complexes, whose DNA footprints are quite similar (65). In relation to the catalytic site, the DNase I footprint of the leading edge of the U138 complex is shifted upstream by $\sim$5 bp compared with the G135 and G143 complexes. Mammalian RNA polymerase II U138 complexes, like the yeast ternary complexes, are also susceptible to intrinsic transcript cleavage (data not shown). Perhaps the efficiency with which ternary complexes carry out intrinsic transcript cleavage correlates with particular types of structural alterations, such as the mobility of the catalytic center forward or back on the DNA template (46), or the positioning of the RNA transcript within the proposed RNA exit channel (66), or extruded from the ternary complex (58). Clearly all these models make testable predictions for future work.

In addition to the rate of hydrolysis, the particular site of transcript cleavage in the ternary complex can be influenced by protein factors. In U138 ternary complexes, TFIIS-induced cleavage generates RNA products distinct from those produced by the polymerase in the absence of TFIIS. This effect on the site of cleavage is also seen with two factors for E. coli RNA polymerase, GreA and GreB. The actions of these two proteins can be distinguished by the distinct sizes of cleavage products generated in their presence from the same ternary complex (67). A distinction in sizes of released products in stalled versus arrested complexes has been documented in ternary complexes containing mammalian RNA polymerase II as well (4). However, for both the bacterial and the eukaryotic enzymes, a size range of released cleavage products can be found from arrested and stalled complexes (Ref. 4 and this work). Thus, there is unlikely to be a discrete differential between arrested and stalled complexes but rather a continuum of cleavage products determined by the many influences that define a ternary elongation complex. Additionally, the prokaryotic elongation factor NusA can change the site of transcript cleavage within a ternary complex (68).

How TFIIS influences the site of transcript cleavage is not known. A priori the expectation would be that TFIIS would simply stimulate the intrinsic cleavage reaction, and, other than reaction rate, no differences in cleavage pattern or released products would be seen with and without TFIIS. However, the distinct sizes of released cleavage products from stalled complexes generated with and without TFIIS suggest there is another layer of mechanistic complexity. Reines and colleagues (62) have reported that human TFIIS cross-links to the 3'-terminal nucleotide of the transcript in an arrested complex, and thus TFIIS is in close proximity with the RNA, and perhaps the polymerase active site. TFIIS has a low affinity for single-strand nucleic acids in solution. However, if there is a relevant nucleic acid binding domain in TFIIS, it might be “unmasked” upon interaction with the ternary complex (63, 64). It remains to be determined whether the apparent differences in the site of transcript cleavage are the consequence of direct interaction of TFIIS with RNA in the ternary complex or a conformational alteration induced by TFIIS upon binding to RNA polymerase. TFIIS also can induce the cleavage reaction when purified RNA polymerase II is bound to purified RNA in the absence of DNA, and the polymerase can then add nucleotides to the newly formed 3'-end (6). Thus, the TFIIS stimulation of the cleavage reaction does not require a ternary complex, and can operate directly on the polymerase when bound to RNA.

2 J. Burt and M. J. Chamberlin, personal communication.
intrinsic cleavage is of interest. The elongation factors ELL, TFIIF, and elonc also interfere with TFIIS-stimulated cleavage (71); whether these factors also interfere with intrinsic cleavage is not known. The toxin α-amanitin, a bicyclic octapeptide, could physically interfere with the approach of TFIIS to the active site of the polymerase or the 3'-terminus of the RNA. Alternatively, TFIIS-stimulated cleavage might require a conformational change of the polymerase, which α-amanitin restricts, directly or indirectly, through binding to a region of the polymerase important in transcript elongation and termination (72). Indeed α-amanitin-resistant mutants cluster in a conserved region of the largest subunit of the polymerase; some mutations in this region of the largest subunit of RNA polymerase III enhance transcript cleavage by 10-fold (73).

RNA polymerase II lacking the RPB9-encoded subunit is significantly impaired in its ability to respond to TFIIS for readthrough stimulation (25) and is less responsive to TFIIS-mediated transcript cleavage. However the Rpb9p-deficient polymerase has no distinguishable difference in the rate of intrinsic transcript cleavage within T1α or U138 complexes. Furthermore, the cleavage products released from U138 or T1α ternary complexes containing this mutant polymerase are the same as those released from complexes containing wild type polymerase. Thus, this polymerase subunit is not necessary for catalyzing intrinsic transcript cleavage, nor in the TFIIS-induced repositioning of the site of transcript hydrolysis. However, it appears to transmit the signal from TFIIS to the rest of the polymerase (25, 41).

Curiously, the homologous subunit in RNA polymerase III, C11, also is essential for the intrinsic cleavage reaction carried out by that polymerase (74). However, there is no TFIIS-like cofactor required to stimulate cleavage for RNA polymerase III. Indeed, RNA polymerase III carries out this reaction in vitro under physiological conditions in the absence of an additional protein factor.

The mutant polymerase, RP11Δ9, possesses “wild type” intrinsic cleavage activity (this work) and binds TFIIS efficiently, yet RP11Δ9 responds poorly to TFIIS as assayed by either cleavage or readthrough (22). These results suggested that Rpb9p transmits the signal from TFIIS to the active site of the polymerase rather than impacting the intrinsic ability of the polymerase to cleave the nascent transcript. For both the wild type polymerase and RP11Δ9, intrinsic cleavage was sufficient to promote readthrough. However, some mutants in TFIIS that stimulate cleavage in arrested ternary complexes nonetheless do not promote readthrough (25, 35, 40). The effect of these mutants led to the hypothesis that TFIIS not only stimulates cleavage but also promotes an additional conformational change needed for efficient readthrough. Clearly this work indicates that the intrinsic cleavage event itself is sufficient to allow readthrough, although we cannot rule out that elevated pH may affect some other feature of the polymerase in addition to stimulating its cleavage activity. However, there were distinct differences observed between intrinsic and TFIIS-stimulated cleavage. First, within stalled but active elongation complexes, the pattern of intrinsic cleavage differed from that generated by TFIIS. In contrast, arrested complexes responded similarly in the intrinsic and the TFIIS-stimulated reactions. The specific molecular distinctions between the stalled and arrested complexes await discovery, and these distinctions will likely explain the differences seen here. Second, the drug α-amanitin, which blocks the translocation step of elongation but not the polymerization step, had little to no effect on the intrinsic cleavage reaction in stalled or arrested complexes yet strongly inhibited TFIIS-dependent reactions.

How have the properties of the intrinsic cleavage reaction reported here and elsewhere (20, 21) informed the elongation mechanism of RNA polymerase? First, the results reinforce the kaleidoscope of potential differences among ternary elongation complexes. It is difficult to define “rules” that pertain always to arrested complexes or always to stalled complexes re: kinetics or released products from intrinsic or factor-induced cleavages. The potential flexibility of regulatory targets is significant for the cell but difficult for the experimentalist. Second, for yeast RNA polymerase II, the subunit encoded by RPB9 is not a major contributor to the transcript cleavage reaction. This input was a possibility because of the phenotype of cells without this subunit and because of the biochemistry of transcription reactions with polymerase lacking this subunit. This subunit clearly remains a mediator between the cleavage stimulatory factor TFIIS and the remaining subunits of the polymerase. Although Rpb9p and TFIIS bind in distinct locations on RNA polymerase II,3 they share very similar regions of amino acid sequence that contribute to elongation stimulation (41). However, the intrinsic transcript cleavage reaction itself is clearly unaffected by the absence of Rpb9p.

The plethora of recently reported crystal structures allows predictions about regions of the polymerase that are involved in the intrinsic cleavage reaction. These regions are likely targets of the activity of TFIIS. The stability of arrested complexes makes them good candidates for structural work as well. Such structures not only will pinpoint amino acid residues important in transcript hydrolysis by the polymerase but may also provide an explanation for how TFIIS stimulates this reaction.

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