Amanitin Greatly Reduces the Rate of Transcription by RNA Polymerase II Ternary Complexes but Fails to Inhibit Some Transcript Cleavage Modes*

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The mushroom toxin α-amanitin, a bicyclic octapeptide, has long been used as a specific inhibitor of RNA polymerase II (1-3). Calf thymus polymerase II has been shown to bind α-amanitin very tightly with a stoichiometry of 1:1, a Kd of 10^-9 m and a complex-dissociation half-time of approximately 1% of the normal rate. Amanitin also greatly slows pyrophosphorolysis by elongation-competent complexes. Complexes which are arrested (that is, which have paused in transcription for long periods in the presence of excess NTPs) are essentially incapable of resuming transcription in the presence of α-amanitin. Complexes traversing sequences that can provoke arrest are much more likely to stop transcription in the presence of the toxin. The substitution of IMP for GMP at the 3' end of the nascent RNA greatly increases the sensitivity of stalled transcription complexes to amanitin. Neither arrested nor stalled complexes display detectable S1-mediated transcript cleavage following amanitin treatment. However, arrested complexes possess a low level, intrinsic transcript cleavage activity which is completely amanitin-resistant; furthermore, pyrophosphorolytic transcript cleavage in arrested complexes is not affected by amanitin.

Both our laboratory (8, 9) and others (10) had observed that promoter-initiated RNA polymerase II ternary elongation complexes can form one or more phosphodiester bonds after amanitin treatment. The combination of these results and the recent finding that the RNA polymerase II ternary complex can catalyze phosphodiester bond cleavage as well as bond formation (11, 12) prompted us to perform a detailed reinvestigation of the effects of amanitin on RNA polymerase II elongation complexes. We report here that RNA polymerase II ternary complexes are generally able to continue RNA synthesis in the presence of α-amanitin, albeit at greatly reduced rates. Interestingly, both intrinsic cleavage activity and pyrophosphorolytic cleavage are completely amanitin resistant in arrested complexes. Given the possibility that arrest may result from a retreat of the active site of RNA polymerase away from the 3' end of the nascent RNA (13), these observations suggest that α-amanitin inhibits RNA polymerase II by disrupting the interaction of the enzyme with the 3' end of the nascent transcript. Our findings also lend further support to a model of transcriptional arrest in which an equilibrium exists between catalytically active and inactive states.

MATERIALS AND METHODS

Ribonucleoside triphosphates were obtained from Pharmacia Biotech Inc., except for ITP which was purchased from Sigma. We used ultra-pure (fast protein liquid chromatography-purified) NTPs for transcription reactions with preinitiation complexes and standard purity NTPs for chase reactions. Labeled ribonucleotides, either [α-32P]CTP or [α-32P]UTP at 800 Ci/mmol, were purchased from DuPont NEN, Bio-Gel A1.5 m was acquired from Bio-Rad, and α-amanitin was purchased either from Boehringer Mannheim or Sigma.

Plasmids—All plasmids used in this study were based on pML5A, which contains the adenovirus 2 major late promoter cloned into pUC18. Plasmids pML5A (14), pML5-4NR (15), and pML20-U158 and pML20-U160 (16) have been described in detail. The pML20-U158 plasmid was referred to as pML20-G155 in Izban and Luse (16); the construction of the pML20 precursor for pML20-U158/U160 was described in Izban and Luse (11). We constructed pMLS-MUT3 from pMLS5 by substituting a synthesized fragment having the sequence 5'-GATCCCTTTTTCTCCATTTTA (nontemplate strand) for the 30-nt BamHI-HindIII fragment which begins at +39 downstream of transcription start. The pML16 series plasmids were all built from a common precursor, pML16LNK, which was derived from pML5A by replacing a BsrHI-BamHI fragment, spanning from +13 to +38 relative to the major late promoter, with a synthesized oligonucleotide. The synthesized fragment bore the original sequence between +13 and +15 but changed the remaining nontemplate strand sequence from 5'-GCTGTTGCGGTTGCGGTCTAGA to 5'-CCCTTCCCGGCGGAGCTCCG-GCCCTTG. The new sequence contains unique XmaI and Apal sites. The pML16220 template was assembled from pML16LNK by replacing the Xmal-Apal segment with a 228-nt XmaI-Apal fragment containing the U-free cassette from pGR220 (17) (a gift from C. Kane). Thus, the

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† The abbreviation used is: nt, nucleotide(s).
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In the course of recent experiments we observed an example of amanitin-resistant chain elongation by RNA polymerase II which was much more extensive than those reported previously (8–10). The reaction which sparked our interest was performed on the pML16220 template, which has no T residues on the non-template strand from +21 through +155 (see Fig. 1A). Transcriptions performed in the absence of GTP gave complexes paused at +23 (C23 complexes). These complexes were Sarkosyl-rinsed and a portion were treated with amanitin at 1 μM/ml for 3 min at 37 °C. (This preincubation protocol was used for all amanitin-containing reactions in this study.) We found that amanitin-treated C23 complexes made an average of 11 or 12 bonds in 5 min at 37 °C when incubated with all four NTPs at 1 mM; some complexes synthesized as many as 30 bonds in 20 min under these conditions (Fig. 1A, lanes 1–5). Most of the RNAs made by noninhibited control complexes were too long to resolve on the gel shown in Fig. 1A. RNA synthesis in the presence of the toxin can continue for at least 2 h at 37 °C (Fig. 1B, lane 6).

The patterns of products obtained on the pML16220 template in the presence of α-amanitin was reproducible in many experiments using different batches of amanitin and nuclear extract. Amanitin was reported to have a very slow off-rate from RNA polymerase II at 37 °C (1.2 × 10⁻⁴/s; see Cochet-Meilhac and Chambon (4)), but those experiments were done under different conditions from those we employed. We were concerned that the catalytic activity of the polymerase in the presence of the toxin at 37 °C might reflect cycling of the drug between solution and polymerase, rather than low activity of the polymerase when amanitin is bound. To address this, we repeated the experiment shown in lanes 1–5 of Fig. 1A, except that after the addition of α-amanitin another round of gel filtration was performed on the C23 complexes to remove free amanitin. We found that in a 20-min chase the majority of these C23 complexes behaved identically to those in lane 5 of Fig. 1A; however, about one-third of the complexes transcribed much more...
FIG. 1. RNA polymerase II complexes still elongate in the presence of α-amanitin but at a reduced rate. A, C23 complexes on the pML16220 template (lanes 1–5) or on pML16220 variants having a T (pML16T27; lanes 6–10) or a C (pML16C27; lanes 11–15) at position +27 on the nontemplate strand were pretreated with α-amanitin as indicated and chased with 1 mM NTPs for the times specified. The initial transcription reaction contained 2 mM ApC and 0.5 mM \(^{32}\)PCTP. B, C23 complexes on the pML16220 template were amanitin-treated and chased with 1 mM NTPs as indicated. The initial transcription reaction contained 100 mM ATP and 1 mM \(^{32}\)PUTP. C, C23 complexes prepared as in B were chased with or without amanitin using the times and NTP concentrations indicated. For all panels, the RNA products were purified and resolved on 20% polyacrylamide gels as described under “Materials and Methods.” Pertinent transcript lengths generated on pML16220 are presented together with template sequence on the left of panel A and on the right of panel B; lengths of various transcripts produced on pML16T27 and pML16C27 are presented together with the template sequence on the right of panel A.
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Amanitin could slow chain elongation either by affecting bond formation itself or by retarding the ability of the active site to translocate along the template. If translocation was the primary target of amanitin, and if complexes stalled by NTP starvation were already poised to add the next base (that is, if translocation of the active site had already taken place), then addition of the initial base in the presence of amanitin would proceed at the normal rate. We tested this idea with C23 complexes produced on the template used in Fig. 1B. While the majority of uninhibited control complexes left the starting position and added several nucleotides within 15 s, amanitin-treated complexes made no bonds in 15 s. The majority of the treated complexes had made no bonds even after 60 s (data not shown). This is consistent with the idea that amanitin blocks bond formation, but it is also possible that stalled complexes must first translocate the active site in order to add the next NTP, in which case translocation could be the step affected by amanitin.

α-Amanitin Exacerbates the Arrested Condition—Many laboratories have observed (11, 22–25) that transcription through certain DNA sequences causes a fraction of the RNA polymerase II ternary complexes to pause without termination. These arrested complexes resume RNA synthesis very slowly (i.e. from 10s of minutes to hours) in the presence of NTPs alone; however, the transcriptional competence of arrested complexes is rapidly recovered by treatment with elongation factor SII (11, 22, 24, 25). We have shown that on the pML5-4NR template arrest occurs 194 bases downstream of the transcription start, within a stretch of T residues on the nontemplate strand (16). This observation is reproduced in Fig. 3. Complexes were arrested at +194, and NTPs were removed by gel filtration (lane 1). These U194 complexes resumed elongation rapidly in the presence of SII and NTPs (lane 5; see also Izban and Luse (16)). In the absence of SII, a much longer time was needed to clear the arrest site (lanes 6–8). Note that the chases in lanes 6–8 were performed with ATP, CTP, and UTP only; we presume that the slow leakage through G-stops at +207 and beyond resulted from GTP contamination in the other nucleotides. When the arrested complexes were challenged with NTPs after amanitin treatment, essentially no resumption of transcription from position +194 was seen, even after 2 h (lane 11). Thus, in contrast to complexes stalled by NTP starvation, arrested complexes are inactivated for further chain elongation by α-amanitin, at least over a time course of several hours. Again, this result was anticipated; since the rate of escape from arrest by resumption of bond formation is normally very slow and bond formation rates are drastically reduced with amanitin, there should be essentially no detectable escape from arrest in the presence of the toxin over the reaction times we employed.

While the arrested complexes cannot continue RNA synthe-
sis in the presence of amanitin, the results in Fig. 3 strongly
suggest that transcript cleavage in these complexes is not
sensitive to the toxin (compare lanes 3 and 4, or lanes 7 and 8, or
10 and 11). We will consider the question of amanitin’s effect on
transcript cleavage in more detail in a later section.

α-Amanitin Increases the Likelihood That Complexes Prone
To Arrest Will Fall into That Condition—Our laboratory previ-
ously showed that complexes stalled after adding a poly(U)
segment (U tail) to the end of the nascent RNA behave progres-
sively more like arrested complexes as the U tail is lengthened
(16). Complexes stalled with a 3’ end consisting of 3 U residues
resumed elongation after a 2-min incubation with excess NTPs.
However, 40% of complexes with an otherwise identical nascent
RNA having 5 U residues at the 3’ end did not resume RNA syn-
thesis when chased for 2 min (16). We used the same
templates employed in the earlier study to investigate the
effect of α-amanitin as a function of the length of the U tail.
Sarkosyl-rinsed complexes were chased to the end of a U-free
cassette at +155 on templates in which either the next 3
(pML20-U158) or the next 5 (pML20-U160) residues on the
non-template strand are Ts (see Fig. 4). The G155 complexes
were then gel-filtered and challenged with various combina-
tions of NTPs, with or without α-amanitin. Most complexes on
both templates resumed elongation from +155 when chased
with all NTPs (Fig. 4, lanes 3 and 13), or when chased to the
G-stop at +159 (lane 4) or +160 (lane 8). The large majority of
C159 complexes on the U158 template resumed transcription
after adding G and incubating for 5 min at 37 °C (lane 6), but
less than half of those starting from +160 on the U160 tem-
pate did so (lane 10), as expected from previous studies (16). It

is important to note that the sequence immediately down-
stream of +159 on the U158 template and +160 on the U160
template is the identical DNA segment, containing only pu-
rines on the non-template strand, which is present downstream
of +23 on the template used in Fig. 1. Thus, one would
expect that C159 complexes on the U158 template and U160
complexes on the U160 template should chase effectively in 5
min in the presence of α-amanitin. However, most of the C159
complexes and nearly all of the U160 complexes failed to re-
sume elongation after amanitin treatment (compare lanes 4
and 5, and lanes 8 and 9). If the complexes were advanced
further along the template before amanitin addition, such that
the 3’ ends of the nascent RNAs were no longer U-rich (com-
plexes G163 on the U158 template and G164 on the U160
template), elongation in the presence of amanitin was once
again efficient (lanes 6 and 7, and 10 and 11). These results
suggest that the effect of amanitin depends not only on the
sequence of bases to be added to the RNA but also on the
sequence at the 3’ end of the nascent transcript. It is worth
noting that in the presence of α-amanitin RNA polymerase II
synthesized the polyuridine segment of RNA between, for ex-
ample, +160 and +172 on the U158 template about as rapidly
as it synthesized the nearly identical RNA (between +24 and
+35) on the template in Fig. 1. Thus, the ability of transcrip-
tion to proceed at a greatly reduced rate in the presence of
amanitin is not strongly affected by the distance downstream
of transcription start.

The Sequence Composition at the Nascent Transcript’s 3’ End
Is a Crucial Determinant of α-Amanitin Inhibition—To further
explore the role of the transcript in amanitin inhibition, we
performed an experiment in which we could compare the aman-
itin response of transcription complexes that differed only in
the 3’ ends of their nascent transcripts. The first G residues
downstream of transcription start on the non-template strand
of the pML16220 template (see Fig. 1) occur at positions +24
through +26. Thus, incubation of C23 complexes with either
GTP or ITP should generate G26 or I26 complexes. The differ-
ent mobilities of the G26 and I26 transcripts confirmed that
IMP was successfully incorporated in place of GMP (Fig. 5,
compare lanes 5 and 6). As expected from the results shown in
Fig. 1, the G26 complexes showed substantial chain elonga-
tion in 5 min in the presence of α-amanitin (lane 11). However,
the I26 complexes, most of which chased in the absence of amanitin
(lane 8), were inactive in a 5-min elongation reaction in the
presence of the toxin (lane 9). This was not the result of a block
of ITP incorporation by amanitin, since the first two bases
added to the +26 complexes are A residues (see Fig. 1A). Note
also that the initial chase from +23 to +26 could be completed
with ITP in the presence of amanitin (lane 3). Thus, transcrip-
tion complexes which are identical except for the three residues
at the 3’ end of the nascent RNA can have very different
responses to α-amanitin.

α-Amanitin Greatly Reduces the Rate of Pyrophosphorolysis
in Stalled Complexes—Stalled RNA polymerase II transcrip-
tion complexes incubated with pyrophosphate liberate NTPs by
sequential cleavage of NMPs from the 3’ ends of the nascent
RNAs (13, 26). Stalled and arrested ternary complexes can also
deave their nascent transcripts without the addition of pyro-
phosphate or other factors (11, 12, 27). We believe this repre-
sents an intrinsic activity of the RNA polymerase and not
residual contamination with SII; this point will be considered
in detail under “Discussion.” Complexes stalled at +20 on the
pML20 template were incubated for 2, 15, or 60 min either with
Mg2⁺ alone or with Mg2⁺ and 2 mM pyrophosphate; for each
condition reactions were performed with or without α-amanitin
(Fig. 6). After 2 min, substantial pyrophosphorolysis was ob-
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**Fig. 4. Sensitivity to α-amanitin increases when the transcript ends with many U residues.** G155 complexes were prepared on either the pML20-U158 (lane 1) or pML20-U160 (lane 15) templates; the initial transcription reactions contained 2 mM ApC and 0.5 μM [α-32P]CTP. Complexes were either incubated with 8 mM MgCl₂ alone for 14 or 16 min (lanes 2 and 14, respectively), chased with 100 μM each of all NTPs for 5 min (lanes 3 and 13, respectively) or walked forward in 3-min incubations, first with 100 μM UTP (lanes 8–11) or with 100 μM each of UTP and CTP (lanes 4–7), next with 100 μM ATP (lanes 6, 7, 10, and 11), and finally with 100 μM ATP (lane 12). Aliquots of complexes stalled at the positions indicated were preincubated with amanitin and then chased with 100 μM each of all NTPs for 5 min (lanes 5, 7, 9, and 11). RNAs were resolved on a 7% polyacrylamide gel. Part of the RNA sequence encoded by each of the two templates is shown along the lower margin, and pertinent transcript lengths are displayed in the left margin.

observed, compared with the control, which received only Mg²⁺ (lanes 2 and 3), and this reaction was nearly completely amanitin-sensitive (compare lanes 4 and 5). After 15 min, however, it was clear that some pyrophosphorolytic cleavage did take place above the Mg²⁺-only background in the presence of amanitin. Quantitation of the remaining 20-mer indicated that only 46% as much uncleaved 20-base transcript remained in lane 9 compared to lane 8. Thus, as we observed with the forward reaction, pyrophosphorolysis in stalled complexes was strongly but not completely inhibited by amanitin. Cleavage in the Mg²⁺-only case was also reduced by amanitin. After 2 min, cleavage was completely blocked (compare lanes 3 and 4); after 15 min, some cleavage had taken place in the presence of amanitin (lane 8) but the amount of uncleaved transcript in lane 7 (no amanitin) was only 51% of the amount in lane 8, indicating that transcript cleavage in the Mg²⁺-only reaction was not completely amanitin-sensitive. Note in lanes 11 and 12, where cleavage with Mg²⁺ had continued for 1 h, that amanitin reduced the total amount of cleavage (the ratio of 20-mer in lanes 11 and 12 was 0.44), and it also strongly reduced the production of the 19-mer (compare also lanes 7 and 8 from the 15-min reaction). However, the production of cleavage products shorter than 19 was actually greater in the presence of amanitin after 1 h, even though total cleavage, as judged by the amount of 20-mer remaining, was reduced. We are not certain of the reason for this. It suggests that amanitin can inhibit only the initial spontaneous cleavage in a stalled complex; once this cut is made, subsequent cleavages are resistant. It is possible that the 19-mer is a metastable species. If cleavage in the presence of amanitin must bypass the 19-mer, subsequent cleavage events may be easier.

Finally, SII-mediated transcript cleavage in amanitin-treated stalled elongation complexes was completely blocked by the toxin in all cases (data not shown), in agreement with earlier results (11).

α-Amanitin Inhibits Neither the Intrinsic Cleavage Activity nor Pyrophosphate-mediated Transcript Cleavage by Arrested Ternary Complexes—We have shown that complexes arrested at +194 on the pML5–4NR template cleave 7–17 nt from the 3′ ends of their nascent RNAs in the presence of SII (13, 16). The same set of 7–17 nt RNAs are released at a much slower rate when the U194 complexes are incubated with Mg²⁺ alone (13). Pyrophosphatase treatment of U194 complexes also results in the relatively rapid release of the 7–17 nt RNAs; in this case the liberated fragments have 5′-triphosphate termini (13). As expected from previous reports (11, 12), SII-mediated transcript cleavage in U194 complexes was completely blocked by α-amanitin (data not shown). The results in Fig. 3, however, indicated that amanitin had no effect on the intrinsic transcript cleavage activity of arrested complexes. A 2-h incubation of +194 complexes with Mg²⁺ gave the same level of truncation products in the presence or absence of amanitin (Fig. 3, lanes 3 and 4). The major shortened transcripts in these lanes appeared to correspond to the major 10- and 14-base cleavages obtained within minutes in SII-mediated truncation reactions of the 194-nt transcript (data not shown for this figure; see Fig. 7B and Rudd et al. (13) and Izban and Luse (16)). As expected (11, 12), transcript cleavage in arrested complexes led to reacquisition of elongation competence, so that intrinsic cleavage in the presence of amanitin, ATP, CTP, and UTP resulted in elongation up to the first G-step upstream of the arrest site, at position +186 (Fig. 3, lanes 10 and 11).

To confirm that amanitin has no effect on the endogenous cleavage reaction, we decided to examine directly the fragments released from the 3′ ends of the nascent RNAs. We prepared U194 complexes whose nascent RNAs were uniformly labeled with [32P]UTP and incubated them for 60 min in Mg²⁺, with or without α-amanitin. RNAs liberated in this reaction were resolved on the gel shown in Fig. 7B. For reference, lane 7 contains RNAs produced by SII-mediated transcript cleavage. As expected (13), lower levels of these same RNAs were obtained in Mg²⁺-only incubations (lane 3). U194 complexes treated with amanitin gave the same level of cleavage products as the noninhibited complexes (compare lanes 3 and 4). Thus, factor-independent transcript cleavage in arrested complexes is amanitin-resistant.

Pyrophosphorolysis in arrested U194 complexes occurred in the presence of α-amanitin (Fig. 7A, compare lanes 2 and 3). However, while the major cleavage at 14 nt from the 3′ end appeared to occur to about the same extent with or without the toxin, other aspects of the cleavage pattern differed between the reactions. Since the initial cleavage produces an elonga-
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...plexes is not sensitive to pyrophosphate. Thus, endonucleolytic transcript cleavage by arrested complex which performs subsequent pyrophosphorolytic cleavages in the absence of the toxin versus a single cleavage in its presence. We resolved this point by examining the short RNA fragments released by incubation of uniformly labeled U194 complexes with pyrophosphate. These RNAs were found to be nearly identical (excepting only a single RNA of about 5 nt) regardless of the presence of α-amanitin in the incubation (Fig. 7B, compare lanes 5 and 6). Thus, endonucleolytic transcript cleavage by arrested complexes in the presence of pyrophosphate is not sensitive to α-amanitin.

DISCUSSION

We have found that the mushroom toxin α-amanitin substantially reduces the rate of transcription by elongation competent RNA polymerase II ternary complexes; however, elongation was not completely blocked. Most amanitin-treated complexes can continue elongation for hours, but complexes which are arrested are essentially unable to resume transcription in the presence of amanitin. While amanitin greatly retards pyrophosphorolysis by elongation-competent complexes, it has no effect on either the intrinsic or pyrophosphate-mediated endonucleolytic transcript cleavage activities of arrested complexes.

It has been observed previously that promoter-initiated RNA polymerase II elongation complexes treated with amanitin can add several nucleotides to their nascent chains (8, 9). Recently, Gu et al. (10) reported that a specifically initiated RNA polymerase II ternary complex stalled at +218 or +220 could continue transcription for about 8 bases in the presence of amanitin. An examination of the sequence of the RNA-like strand of the template used by Gu et al. downstream of +218/220 shows that all but one of the next 9 or 11 bases are purines, followed by three pyrimidines (10). Thus, it seems likely that Gu et al. observed the same effect which we document here. We speculate that the relative rarity of sufficiently long purine runs in random sequence DNA accounts for the lack of previous reports on the incomplete inhibition of transcript elongation by amanitin (but see Job et al. (29), discussed below).

We have reported that RNA polymerase II initiating at the adenovirus 2 major late promoter cannot add even a single nucleotide to a dinucleotide primer in the presence of amanitin (30). In those experiments, production of a low level of trinucleotide was observed with amanitin, but this same level was also seen even in the absence of template, or with dinucleotides that could not prime RNA synthesis at the adenovirus promoter. We interpreted these results to mean that the amanitin-resistant trimer was generated by activities other than RNA polymerase II. However, in light of our current results we cannot exclude the possibility that a very low level of transcription initiation can take place in the presence of amanitin. RNA polymerase II preinitiation complexes assembled from nuclear extracts are unstable in the presence of ATP, which is required for transcription initiation under these conditions (30, 31).
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Thus, amanitin-treated preinitiation complexes might be inactivated before any bonds could be formed.

The effects of amanitin on transcription of homopolymeric templates by pure RNA polymerase II have also been studied. Two groups reported that α-amanitin permits formation of the first phosphodiester bond but absolutely blocks the synthesis of all subsequent bonds (32, 33). It is difficult to compare these results to our own findings since the RNA polymerases in these systems did not pass through initiation at a promoter. One study on transcription of homopolymeric templates detected extensive amanitin-resistant transcription by polymerase II. Job et al. (29) reported that with poly(dC) or poly(dC)-poly(dG) as template and GTP as substrate, about 30% of RNA synthesis by wheat germ RNA polymerase II was resistant even to 100 μg/ml α-amanitin. Slippage of the nascent transcript on the template clearly played a part in these results (29), but it is interesting that the only template-substrate combination which allowed significant amanitin-resistant transcription involved the synthesis of a polypurine transcript.

We found that the sequence of the region to be transcribed was a major factor in the ability of RNA polymerase II to extend the nascent RNA, in the presence or absence of amanitin (Fig. 1, A-C). However, the results of substituting ITP for GTP in only the last three bases of the nascent RNA (Fig. 5) argue that the sequence of the transcript is also important in elongation. In order to rationalize these observations it is useful to briefly review current ideas on the mechanism of transcriptional arrest. Several groups have noted that arrest by RNA polymerase II most often occurs immediately after the synthesis of a U-rich RNA (20, 22, 23, 34–38). Sequences flanking the template region encoding the poly(U) segment also play a very important role in arrest (23, 34, 39, 40). We had noted that DNA with 5 consecutive T residues on the nontemplate strand does not provide a barrier to the polymerase during transcription with excess NTPs. However, if the polymerase was forced to pause after the incorporation of the 5 U residues, because of the absence of the next NTP required for elongation, then nearly half of the complexes could not resume transcription after a 5 min incubation with excess NTPs (16). Thus, while the incorporation of many consecutive U residues does not necessarily force arrest, polymerases crossing T-rich sections of the nontemplate strand are in danger of arrest. Our results suggested that the length of time which the polymerase spends with a transcript containing a U-rich 3' end is crucial to the arrest process. Very recently, the importance of “dwell time” at potential arrest sites was directly assessed by changing the overall rate of transcription with TFII F or ammonium ions; in both cases, more rapid transcription was inversely correlated with arrest (41).

Once arrest has occurred, rapid resumption of transcription cannot take place without cleavage of the nascent RNA well upstream (from 5 to 6 as many as 17 bases) of the initial site of bond formation (16, 42). Although transcript cleavage occurs spontaneously in both stalled and arrested complexes, it is greatly stimulated by the SII elongation factor (11–13). The source of this spontaneous cleavage is still somewhat controversial. We have argued that this activity is intrinsic to the RNA polymerase itself. While we cannot absolutely eliminate the possibility of SII contamination in our partially purified complexes, the following points argue strongly against it. First, if the cleavage were caused by a very low level of residual SII, which was not removed by gel filtration in the presence of Sarkosyl, one would expect that a second round of gel filtration under the same conditions would remove almost all of the residual activity. However, when we did such an experiment, we did not see any reduction in cleavage levels after the com-
plexes had been subjected to a second gel filtration step (data not shown). Second, the factor-independent cleavage activity in Fig. 6 makes its initial cut only one nucleotide from the 3' end; however, the addition of SII to stalled complexes leads to cleavage primarily in dinucleotide increments (26). The action of amanitin on arrested complexes also argues against contamination. We can see no stimulation of cleavage by added SII in the presence of amanitin, and yet the spontaneous cleavage activity is completely amanitin-resistant. This might be explained if amanitin could only inhibit the binding of SII; in this model, residual SII, which is already bound, would not be inhibited. However, when we tested this idea by adding SII to complexes and then followed with amanitin, we still saw absolutely no cleavage above the control (data not shown). Finally, it is important to recall that Escherichia coli RNA polymerase shows spontaneous transcription cleavage activity even when it is prepared from cells which lack functional genes for both the GreA and GreB transcript cleavage factors (43). RNA polymerase III, which has no known elongation factors, also exhibits spontaneous cleavage of transcripts in stalled complexes in the presence of Mg$^{2+}$ (44).

We had hypothesized that arrest might result from loss of contact between the active site of the polymerase and the 3' end of the transcript (16). Transcript cleavage was seen as a mechanism to generate a new 3' end that is accessible to the active site. The subsequent demonstration that pyrophosphate can also stimulate cleavage at the same sites as SII suggested that the active site itself might be the cleavage agent (13). Arrest could then reflect the translocation of the RNA polymerase's catalytic center upstream along the nascent RNA. It is plausible that U-rich regions are the least avidly bound by the active site, making transcription complexes with U-rich 3' ends the most prone to arrest. Elongation competence would be restored by SII-stimulated cleavage at upstream locations on the transcript with which the active site stably associates.

In the context of this model (see also Gu and Reines (41)) the importance of dwell time at potential arrest sites is easy to envision. If upstream translocation of the active site is much slower than the usual rate of bond formation, arrest will be very unlikely unless the polymerase can be paused for some time after synthesis of the crucial U-rich 3' end. Thus, in Fig. 4, a C159 complex whose transcript ends ...GUUUUC-3' is mostly active when chased, but when amanitin is added to greatly increase dwell time (by lowering the rate of initial bond formation), most of the C159 complexes are inactive upon chase (compare lanes 4–6). A U160 complex, with a more U-rich 3' end (...GUUUUUC-3'), is only partially active in the absence of amanitin and nearly inactive in the presence of the toxin (compare lanes 8–10 of Fig. 4). We had suggested (16) that the active site probably partitions between elongation-competent and elongation-incompetent locations in arrested complexes, since arrested complexes show a very slow but easily detected rate of resumption of transcription (Fig. 3, lanes 6–8). Bond formation at 37°C and 1 mM NTPs occurs on average about 5 times/s (20), so the active site would need to be in the elongation competent configuration for only a very short period to allow some complexes to escape from arrest. However, bond formation is much slower in the presence of amanitin, which would account for the inability of amanitin-treated, arrested complexes to resume elongation (Fig. 3).

What conclusions can we draw concerning the mechanism of inhibition of transcription by amanitin, given both our present results and the large body of earlier work on the toxin? Previous studies with homopolymeric templates (29, 32, 33) had suggested that translocation and not bond formation is blocked by amanitin, since the initial bond can be formed when amanitin is present but transcription cannot continue. As we noted above, we cannot discriminate between these models from our own results. Johnson and Chamberlin (45) showed that binary complexes of yeast RNA polymerase II and RNA could not only cleave the RNA but could also add nucleotides to the newly-created 3' ends. This template-independent bond addition was characterized as partially sensitive to amanitin. It is difficult to envision how amanitin functions to inhibit translocation along the template when it also affects bond addition in a template-independent reaction. From our own work, we can say that amanitin does not "tie down" the active site, since amanitin-treated arrested complexes can cleave their nascent RNAs at locations far upstream of the original polymerization site in the presence of amanitin. However, this result could still be obtained if amanitin blocks downstream, but not upstream, translocation of the active site.

Perhaps the most interesting aspect of our results is the fact that amanitin does not inhibit several of the catalytic activities of the RNA polymerase. It is striking that amanitin inhibition occurs only when the active site is near the 3' end of the transcript, with the exception of SII-mediated cleavage in arrested complexes. Spontaneous cleavage and pyrophosphorylation in arrested complexes are both completely insensitive to amanitin. This suggests that amanitin must work through the 3' end of the transcript, or alternatively, that amanitin binds near a location normally occupied by the 3' end of the RNA. Such an idea is consistent with the findings of Johnson and Chamberlin (45), who showed that amanitin does not inhibit the initial SII-mediated cleavage reaction in binary complexes, when the active site presumably occupies an internal position on the transcript. However, amanitin does block any further cleavage in these complexes. Note that after the initial cleavage in binary complexes the active site must be at the 3' end of the RNA, since bond formation can occur after the first cleavage (45). Mutations have recently been described in the largest subunits of E. coli (46) and Bacillus subtilis (47) RNA polymerases which confer resistance to streptolydigin. These mutations occur in region F (48), a segment which shows considerable sequence similarity among the largest subunits of both eukaryotic and prokaryotic RNA polymerases. Region F is also the location of amanitin resistance mutations in RNA polymerase II (see Bartolomei and Corden (49) and references therein), which is not unexpected since streptolydigin's effect on pyrophosphorylase parallels the effect of amanitin on RNA polymerase II. Significantly, it has been proposed that region F might form part of the binding site for the 3' end of the nascent RNA (48).

The fact that amanitin blocks SII-mediated transcript cleavage in arrested ternary complexes would seem to violate the idea that amanitin can act only near the 3' end of the transcript. However, it is possible that amanitin simply blocks access of SII to the upstream sites at which transcript cleavage takes place, rather than blocking the cleavage reaction directly. This is again consistent with the binary complex results (45). Binary complexes are probably less sterically confined than ternary complexes, and as just noted the initial SII-mediated cleavage in binary complexes is not amanitin-sensitive.

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