Hydrolytic Cleavage of Nascent RNA in RNA Polymerase III Ternary Transcription Complexes*

Highly purified yeast RNA polymerase III ternary complexes were found to possess a hydrolytic chain retracting activity that cleaves nascent RNA from its 3'-OH end. Most of the shortened transcripts were capable of resuming RNA chain elongation, indicating that they remain stably associated with the enzyme-DNA complex. Analysis of the products of cleavage indicated that retraction primarily occurred in dinucleotide increments, but that mononucleotides were also excised at lower frequency. The ribonuclease activity was totally dependent on the presence of a divalent cation and was stimulated by the addition of non-cognate ribonucleotides. The inclusion of ATP in the reaction enhanced both the rate and extent of transcript cleavage. Evidence suggesting that the hydrolytic activity is intrinsic to RNA polymerase III and factor-independent is also presented. Transcript cleavage by RNA polymerase III ternary complexes appears to be more closely related to the intrinsic nucleolytic activity of vaccinia virus RNA polymerase ternary complexes than to TFIIS-dependent cleavage that has been described for RNA polymerase II ternary complexes.

Elongation of RNA chains in transcription is catalyzed by RNA polymerase within a highly processive enzyme-RNA-DNA ternary complex. The substrates for elongation are ribonucleoside triphosphates that are sequentially transferred to the 3'-OH end of the nascent RNA through the formation of 3'-5'-phosphodiester linkages and the release of pyrophosphate. The ternary complex also mediates the reverse reaction, pyrophosphorylation, yielding back the substrates of elongation and a shortened RNA chain.

It has recently been demonstrated that a number of different RNA polymerases in ternary complexes have a second RNA chain retracting activity (reviewed in Ref. 1). In this process, retraction of the RNA polymerase along the DNA template occurs through hydrolytic cleavage of the growing end of the nascent transcript, generating short RNA products. It has been postulated that this hydrolytic retraction provides a mechanism for overcoming elongation arrest and that the process in which the paused complex retracts and subsequently resumes elongation facilitates multiple approaches to the block in transcription (2). Eukaryotic RNA polymerases must transcribe DNA that is packaged into chromatin, whose components, principally the nucleosomes, represent potential obstacles to RNA chain elongation. Indeed, there is some evidence to suggest that nucleosomes can provide some impediment to RNA chain elongation by RNA polymerase (pol) III (3, 4) and more so for pol III (5, 6). Sequence-specific DNA-binding proteins may also interfere with the passage of RNA polymerase along the template. DNA-bound Escherichia coli lac repressor and EcoRI block transcription by E. coli RNA polymerase and pol II (7-9). In addition, elongating ternary complexes have a high probability of becoming trapped at certain sequences within transcription units such that they can neither elongate nor dissociate (reviewed in Refs. 10, 11). The elongation factor TFIIS (also called SII; 12), which interacts with the largest subunit of pol II (13, 14), greatly mitigates transcriptional stalling at such intrinsic arrest sites (15-17). This accessory factor also facilitates readthrough of the obstruction provided by a lac repressor-operator complex (8). Furthermore, TFIIS has been shown to be a determinant (possibly the sole determinant) of the nucleolytic activity in pol II ternary complexes. In the presence of TFIIS, the pol II ternary complex cleaves RNA in a processive 3' → 5' manner, releasing mononucleotides, dinucleotides, and to a lesser extent, trinucleotides and even longer products (18-21). Importantly, there is strong evidence to indicate that this TFIIS-stimulated nucleolytic cleavage is essential for TFIIS-potentiated readthrough past intrinsic or protein-induced blocks to RNA chain elongation (2, 22, 8).

Hydrolytic cleavage is not solely an eukaryotic phenomenon. Indeed, transcript cleavage was first demonstrated in E. coli RNA polymerase ternary complexes (23). Two newly identified elongation factors, GreA and GreB, are responsible for stimulating this cleavage reaction of E. coli RNA polymerase ternary transcription complexes (24, 25). In the presence of GreA, the nascent RNA is cleaved in steps of two or three nucleotides, whereas the action of GreB results in the release of longer (up to nine nucleotides) RNA products (25). Whether nucleolytic RNA cleavage is an intrinsic property of RNA polymerase that is stimulated by accessory factors such as GreA, GreB, and TFIIS, or whether the nucleolytic activity resides in these factors remains a point of uncertainty (1). Here we report the identification and characterization of a divalent cation-dependent 3' → 5'-exonuclease activity associated with highly purified Saccharomyces cerevisiae RNA polymerase III ternary complexes. We find that pol III stalled on a SUP4 template is capable of cleaving nascent RNA in both mono- and dinucleotide steps. Nucleolytic cleavage is stimulated by the presence of non-cognate ribonucleoside triphosphates. We also present evidence that a dissociable, TFIIS-like factor is not involved with nucleolytic RNA chain retraction by pol III.

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1 The abbreviations used are: pol, RNA polymerase; nt, nucleotide(s); TLC, thin layer chromatography; bp, base pair(s); ATPyS, adenosine 5’-O-(thiotriphosphate); AMP-PCP, β,γ-methyleneadenosine 5’-triphosphate; AMP-PNP, β,γ-imidoadenosine 5’-triphosphate; AMP-CP, α,β-methyleneadenosine 5’-triphosphate.
**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled fast performance liquid chromatography purified ribonucleotides (Pharmacia LKB Biotechnology, Inc.), (α-32P)CTP (New England Nuclear), shrimp alkaline phosphatase (U. S. Biochemical Corp.), dinucleotides, and yeast inorganic pyrophosphatase (Sigma) were purchased.

Highly purified RNA polymerase III was prepared as described (26, 27). The DNA template used in this study was pLN4031, which contains the SUP4 tRNA5 gene (28, 29), cleaved with PstI and HindIII, followed by extraction with phenol-chloroform and precipitation with ethanol.

**Purified Terinary Complexes**—Terinary complexes stalled at position C31 (see Fig. 1) were typically formed in 100 reaction mixtures that contained 50–100 fmol of active RNA polymerase III, 50 fmol of PstI- and HindIII-cleaved pLN4031 DNA template, 400 μM GTP, 35 μM ATP, 100 μM UTP, and 5 μM (α-32P)CTP (200,000 counts/min/pmol) in transcription buffer (40 mM Tris-HCl, pH 8, 150 mM MgCl2, 3 mM dithiothreitol, 160 μg/ml bovine serum albumin, 5% glycerol) for 45 min at 20–21 °C. The reaction was terminated by the addition of Na2EDTA to 10 μM followed by gel filtration on a 1-ml Sepharose CL2B column equilibrated in transcription buffer without MgCl2. Terinary complexes were harvested by collecting 2-drop fractions (30). Samples were analyzed (30) by directly spotting aliquots (10 pL) onto Thin Layer Chromatography plates. Ternary complexes contained RNA chains of 10, 12, and 17 nt were identified by counting bands shorter than the C31 initial complex on over-exposed autoradiograms. This indexing was confirmed for transcripts shortened to nt A19 by observing the effect of Mg2+-induced cleavage were identified by counting bands shorter than the C31 transcript was confirmed by the two-nucleotide sequencing screen.

**Thin Layer Chromatography (TLC) of Reaction Products**—Transcript retraction reactions were carried out as described above. Samples were analyzed (30) by directly spotting aliquots (10 μl) onto a polyethyleneimine-coated TLC plate (Sigma) which was developed in either 0.5 or 1% LiCl until the solvent front had migrated approximately 15 cm. The TLC plates were subjected to autoradiography with an intensifying screen.

**Mono- and Dinucleotide Standards**—The CMP standard was produced by mixing 1 pL of 0.065 μM (α-32P)CTP (6000 Ci/mmol) with 3 μl of 0.1 M dithiothreitol. The mix was incubated at 10°C for 30 min. The reaction was then incubated on ice for a further 10 min before the addition of 3 μl of 1 N NaOH.

Dinucleotides (UpC, CpU, AgC) were 5'-labeled in 30-μl reactions containing 100 μmol of NpN, 5 units of T4 polynucleotide kinase (U. S. Biochemical Corp.), and 12.5 μmol of 32P-PIATP (6000 Ci/mmol) in 70 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 5 mM dithiothreitol. The reactions were incubated at 37°C for 30 min, cold ATP was added to 6 μM followed by incubation at 37°C for a further 30 min. All standards were diluted in column buffer (40 mM Tris-HCl, pH 8, 100 mM NaCl, 3 mM dithiothreitol) before use.

**RESULTS**

Purified pol III is capable of initiating transcription site specifically and efficiently at 3'-overhanging DNA ends generated by restriction endonuclease cleavage. We have used this property of pol III to examine whether it contains an intrinsic 3'-to-5' ribonucleic acid activity, as previously observed with vaccinia virus RNA polymerase (31), or whether a separable elongation factor like TFIIIS for pol II or GreA and GreB for E. coli RNA polymerase is required for efficient RNA chain retraction (22, 25).

Terinary complex (pol III-DNA-RNA) transcription complexes contain-

![Fig. 1. Formation of C31 ternary complexes. The 5'-flanking sequence of the SUP4 tRNA5 gene (plasmid pLN4031) is shown extending from the 3'-overhang that is generated by cleavage with PstI to the 5'-proximal box A promoter element. Transcription was primed with GpG (underlined) to initiate at the single strand-double strand junction and elongated with ATP, (α-32P)CTP, and UTP to position C31.](image-url)

By convention, stalled complexes are identified according to the nucleotide at the 3' end and the length of the transcript.
under "Experimental Procedures." C31 complexes were elongated with the times indicated and purified by Sepharose CL2B chromatography, providing 0.1 M~ after a short period of Mg^{2+}-dependent chain retraction extended the 29-nt RNA back to 31 nt (panel b, compare lanes 1 and 2), confirming that the Mg^{2+}-induced shortening had occurred at the 3' end of the nascent chain. A significant fraction of C27 and A26 RNA generated by Mg^{2+} treatment was incapable of resuming elongation after 5 and 30 min of Mg^{2+} treatment, and the proportion of these unchaseable transcripts increased with time (panel a, lanes 9 and 11). The failure of the C27 and A26 RNA to restart elongation resulted from disruption of the complex upon Mg^{2+}-induced chain retraction to these positions. Rechromatography of complexes on CL2B after a 5-min incubation in the presence of MgCl_{2} and 2.85 units of pyrophosphatase; lane 5, 7 mM MgCl_{2} and 2.85 units of pyrophosphatase; lane 6, 7 mM MgCl_{2} and 1 mM pyrophosphate; lane 7, 7 mM MgCl_{2}, 1 mM pyrophosphate, and 2.85 units of pyrophosphatase.

In experiments not shown, transcript shortening by the pol III ternary complex was observed over a broad range (1–50 mM) of Mg^{2+} concentrations. Furthermore, other divalent cations could substitute for Mg^{2+} in supporting this cleavage reaction: retraction in the presence of 7 mM Mn^{2+} resulted in a similar distribution of shortened RNAs to that observed with Mg^{2+} after 5 min of incubation (that is to C29 and C27 in comparable amounts), while Zn^{2+} or Co^{2+} addition to 7 mM yielded transcripts predominantly shortened by two nucleotides but not significantly more. A U30 transcript was also observed upon addition of Mg^{2+} to 29-nt RNA chains had evidently released since they eluted in the included volume (data not shown).

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nucleotides on RNA cleavage within the pol III ternary complex (Fig. 4). In these experiments nucleotides were added to a final concentration of 1 mM. The presence of ATP had a marked effect upon transcript truncation, increasing the extent of retraction, with A26 and A24 RNA becoming the predominant species (panel a, lane 4). UTP also influenced the extent of retraction and the distribution of transcripts, generating mainly RNA species U28, A26, and U25 (lane 5). By comparison, CTP addition decreased the appearance of shorter transcripts, and RNA shorter than C27 was not detectable (lane 6). This result is consistent with retraction occurring in mononucleotide increments in which removal of the 3′-CMP residue would be accompanied by reincorporation of CMP. However, the presence of low levels of UTP, due to deamination of CTP, also would generate this result if retraction occurred in dinucleotide increments followed by reincorporation of both CMP and UMP. GTP addition allowed the stalled ternary complex to extend the RNA chain to position G33 as predicted by the sequence (lane 7). Addition of each nucleotide in the absence of Mg²⁺ had no detectable effect on chain retraction (lanes 8–11).

In contrast to the disruption of ternary complexes that occurred upon Mg²⁺-induced retraction to C27 and A26, complexes that retracted to A24 (and C29) upon addition of Mg²⁺ and ATP (Fig. 4b, lane 1) remained predominantly intact; addition of ATP, CTP, and UTP extended the transcript back to C31 (lane 2) and addition of all four NTPs generated full-length product (lane 3). ATP-enhanced retraction, nevertheless, resulted in mostly inactive RNA at positions U28, C27, and A26 (compare lanes 2 and 3 with 1). If the presence of the cognate substrate) ATP stabilized complexes retracted to A24 (against reiterative retraction occurring in increments of one to three nucleotides to U21, A22, and A23), it clearly was unable to do so at position A26 (where ATP is the only cognate nucleotide when retraction occurs in mononucleotide increments to U25). Thus, when retraction of two or seven nt occurred, the transcript remained associated with pol III, whereas retraction of three, four, or five nucleotides led to the formation of inactive complexes. Evidently it is not simply the case that pol III releases the transcript after retracing its path along DNA by more than two nucleotides. Indeed, pol III may undergo conformational changes during retraction that results in the formation of either stable or metastable ternary complexes depending upon its position on the template.

Investigation of the kinetics of retraction showed that ATP increased the rate of RNA cleavage, both in regard to the extent of truncation and the proportion of the C31 complex converted to shortened RNA (Fig. 5; compare 0.26 min ± ATP). Surprisingly, the addition of pyrophosphate to 1 mM substantially reversed the stimulatory influence of ATP, such that the rate of transcript shortening was reduced and the distribution of retracted transcripts reverted toward that observed in the presence of Mg²⁺ alone. This pyrophosphate-generated effect was not observed when inorganic pyrophosphatase was also included in the reaction mixture, supporting the contention that the phenomenon was due to pyrophosphate (data not shown). While the molecular basis for pyrophosphate-mediated inhibition of ATP-enhanced chain retraction is not clear, it resembles the effect of pyrophosphate on transcript truncation by the combined action of pol II and TFIIS (19).

In order to assess whether ATP hydrolysis was involved in ATP-enhanced retraction, dATP, ATP-γS, AMP-PCP, AMP-PNP, AMP-PPi, ADP, and AMP were substituted for ATP at a final concentration (data not shown). ATP-γS substituting efficiently for ATP, all other nucleotides except AMP and dATP

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**Fig. 4. Effect of ribonucleotides on retraction by pol III.** Panel a, C31 ternary complexes were column isolated (lane 1), to which individual nucleotides (indicated above the lane) at 1 mM final concentration were added either with (lanes 4–7) or without (lane 8–11) 7 mM MgCl₂, and incubated for 5 min or immediately chased by the addition of 0.1 mM NTP and 7 mM MgCl₂ (lane 2). Panel b, ATP-enhanced retraction of C31 ternary complexes was performed by adding 1 mM ATP and 7 mM MgCl₂ and incubating for 5 min (lanes 1–3). Transcript cleavage reactions were subsequently tested for retention of RNA chain elongation activity by adding ATP, UTP, and CTP (lane 2) or all four NTPs (lane 3) and incubating for 2 min. All chase nucleotides were at 0.1 mM final concentration, except for ATP which was approximately 1 mM, as a result of its inclusion in the transcript cleavage reaction.

**Fig. 5. Influence of ATP and pyrophosphate on the kinetics of transcript cleavage.** Retraction was performed by incubating C31 ternary complexes (unmarked lane) with 7 mM MgCl₂ for the times indicated, either alone, with ATP (1 mM), or with ATP (1 mM), and pyrophosphate (1 mM).
generated weak enhancement of retraction. This weakly enhanced retraction by non-hydrolyzable nucleotides may not necessarily signify that ATP hydrolysis was not required since the same experiments showed that ADP, AMP-PCP, AMP-PNP, and AMP-CPP were all contaminated with ITP (as evidenced by trace extension of the original C31 complex to a larger size). A 1% contamination of ATP would have approximately the enhanced level of retraction observed. The requirement of ATP hydrolysis in enhanced retraction therefore does not appear likely, but it is not excluded at this time.

RNA cleavage by pol II and by E. coli RNA polymerase ternary complexes has been demonstrated to be greatly stimulated by dissociable elongation factors (reviewed in Ref. 1). Although the preparation of pol II used for these experiments was highly purified (27), we could not rule out the possibility that a dissociable factor was responsible for, or stimulated, transcript cleavage. We therefore formed ternary complexes as previously described but added Sarkosyl to 0.3% prior to loading on the Sepharose CL2B column. A similar method has been employed by others to remove elongation factor TFIIS from pol II ternary complexes (22). A major fraction of the ternary complexes isolated by this procedure (Fig. 6, lane 1) remained active and were capable of elongating to full-length (lane 2). Upon presentation with Mg2+, transcript cleavage was detectable after 15 s (lane 3) and was more extensive than that observed for complexes that had not undergone Sarkosyl treatment (Fig. 2, lane 5). A similar experiment in which Sarkosyl was added to 0.3% after column isolation of C31 ternary complexes for 2 min resulted in considerable inactivation of these complexes such that only a small fraction were competent to resume elongation to full-length. We did note, however, that in the presence of 0.3% Sarkosyl equivalent proportions of complexes were capable of retraction and elongation (data not shown).

\( \alpha \)-Amanitin, an inhibitor of RNA chain elongation, has been shown to inhibit RNA chain retraction of RNA polymerase II (34, 22, 19). Although \( \alpha \)-amanitin is not an effective inhibitor of RNA chain elongation for yeast pol III, tagetitoxin is (35). Addition of tagetitoxin to 8000 units/ml abolished production of full-length transcript upon addition of nucleotides to column-isolated C31 complexes, but did not inhibit Mg2+-induced retraction (data not shown). We did note that tagetitoxin appeared to change the register of retraction at this high concentration, such that Mg2+-induced bands corresponding to U30 and U28 were observed in place of C29 and C27. However, product analysis, similar to that described below, demonstrated that addition of tagetitoxin resulted in the misincorporation of a pyrimidine nucleotide prior to Mg2+-induced retraction. Likewise, addition of calf intestinal alkaline phosphatase to a reaction mixture containing tagetitoxin, prior to the addition of MgCl2, but not after, eliminated the tagetitoxin effect (presumably due to the hydrolysis of the contaminating NTP) (data not shown).

We used polyethyleneimine thin layer chromatography (30) to characterize the products of the retraction process. Isolated C31 complexes uniformly labeled with CTP were exposed to Mg2+ to induce transcript truncation, and a portion of the reaction mixture was spotted onto a TLC plate that was developed in 1 M LiCl (Fig. 7a). The failure to find labeled CTP as the reaction product argues against cleavage via pyrophosphorolysis (lane 4). Products co-migrating with CMP and dinucleotide markers were observed instead. We conclude that transcript cleavage in the pol III ternary complex occurs via hydrolytic cleavage rather than by pyrophosphorolysis. The use of 0.5 M LiCl as the solvent allowed greater resolution of mononucleoside monophosphates from dinucleoside diphosphates, predicted to be the hydrolysis products generated by incremental cleavage by one or two nt from the 3'-end of the RNA (note that pUpC and pCpU migrate identically in this system; see sequence in Fig. 1). Analysis of the reaction products in this way revealed that transcript truncation mostly yielded dinucleotides and, to a lesser extent, CMP (Fig. 7b, lane 4; 3'-UMP should migrate faster). Although inorganic phosphate also migrates between CTP and CMP in this system, other data indicate it is not a major hydrolysis product (see below). The inclusion of ATP in the reaction that was shown above to enhance cleavage, led to a decrease in the proportional yield of CMP (lane 5). We conclude that RNA chain retraction by pol III ternary complexes can proceed in single nucleotide and in double nucleotide steps.

We sought to characterize the dinucleotide products further using high resolution polycrylamide gel electrophoretic analysis. It is possible to resolve different species of dinucleotides on 28% polycrylamide gels, the hierarchy of mobility being YpY > YpR/RpY > RpR (20). Thus, while it is possible to distinguish between dinucleotides with different base compositions it is not possible to resolve them by sequence (20). Portions of the reaction mixtures described in Fig. 7, a and b, were examined using this method, and the results are shown in Fig. 7c. Consistent with the polyethyleneimine-TLC analysis, the major products of retraction induced by Mg2+ were found to be CMP and a dinucleotide that co-migrates with pUpC/pCpU (lane 4). As also observed by TLC analysis, ATP reduced the formation of CMP product. A putative dinucleotide species with a slightly lower mobility than pUpC/pCpU was also detectable, but this was present at a much lower level than the major dinucleotide product. Based upon its mobility we do not believe that this represents a trinucleotide. It is possible that this product is the result of pol III retraction past C27, which is seen at a low level in these experiments, or less probably from low level incorporation of IMP at position 32 in the starting reaction with subsequent generation of pCpI.

We also investigated the kinetics of product formation, demonstrating that the pUpC/pCpU dinucleotide is the major product 15 s after the initiation of transcript truncation (Fig. 8, lane 3). A small amount of CMP was also observed at this time. The slower migrating dinucleotide product formed more slowly, becoming detectable after 2.5 min of RNA chain retraction. If this slowly migrating product is pCpI, its rate of excision as the first product to be formed must be slow. Removal of 5'-phosphate from these products with alkaline phosphatase
Fig. 7. Products of transcript shortening. Panel a, Sepharose-purified C31 ternary complexes were retracted with 7 mM MgCl₂ without or without 1 mM ATP for 5 min. A portion of each reaction mixture was spotted onto a TLC plate which was developed in 1 M LiCl. CTP, CMP, pCpU, and pApC were chromatographed as markers. Lane 1, CTP; lane 2, CMP; lane 3, C31 ternary complexes; lane 4, C31 ternary complexes + MgCl₂; lane 5, C31 ternary complexes + MgCl₂ + ATP; lane 6, pCpU; lane 7, pApC. Panel b, as for panel a, except that the TLC plate was developed in 0.5 M LiCl. Panel c, analysis of products on a 28% sequencing gel. Reactions were as described for panels a and b. Lane 1, CTP; lane 2, CMP; lane 3, C31 ternary complexes; lane 4, C31 ternary complexes + 7 mM MgCl₂; lane 5, C31 ternary complexes + 7 mM MgCl₂ + ATP; lane 6, pCpU; lane 7, pApC; lane 8, pApC.

Fig. 8. Time course of product formation. Isolated C31 ternary complexes were retracted with 7 mM MgCl₂ for the times indicated above the lanes (lanes 2-6, 11, and 12). Markers were included as standards as indicated (lanes 1 and 7-10; the dinucleotides CU, AC, and UC were 5'-phosphorylated). Transcript cleavage reactions were also treated with 10 units of shrimp alkaline phosphatase for 2 min at 20 °C (lanes 11 and 12). The sample for lane 12 contains ternary complexes, which were inactivated by heating at 100 °C for 2 min prior to the addition of 7 mM MgCl₂ and 10 units of shrimp alkaline phosphatase. This was used to distinguish between dinucleotides with external and internal label. Since these transcripts were labeled with [α-³²P]CTP, pUpC but not pCpU should have label that is phosphatase-resistant. Removal of the 5'-phosphate from dinucleotide diphosphates has been previously demonstrated to result in a large decrease in mobility of the dinucleotide in this gel system (20, 21). Upon treatment with alkaline phosphatase, most of the dinucleotide-incorporated label remained, but was contained in a much slower migrating compound (lane 11). When C31 complexes were boiled prior to the addition of MgCl₂ and alkaline phosphatase no RNA cleavage products were generated (compare lane 12 with lane 2) indicating that the new product generated in lane 11 was not the result of an endonuclease contaminant in the alkaline phosphatase. These results are consistent with the conclusion that most of the dinucleotide product of 3' → 5' RNA chain retraction contains an internal label and is therefore pUpC. Consistent with this conclusion, when transcripts were labeled with [α-³²P]UTP, most of the label in the dinucleotide retraction reaction was lost upon treatment with phosphatase (data not shown). This result is also consistent with the assumption that the observed dinucleotide cleavage products are 5' rather than 3'-phosphorylated.
since the initial dinucleotide product would be monophosphorylated which is not observed. Based upon our findings, we believe that while retraction can occur in either mono- or dinucleotide steps, the primary pathway for retraction of the pol III C31 ternary complex on the SUP4 tRNA template starts with the hydrolytic cleavage of two pUgC dinucleotides from the 3'-end of the transcript.

**DISCUSSION**

The potential to cleave nascent RNA by a hydrolytic mechanism has recently emerged as a property of several prokaryotic and eukaryotic RNA polymerase ternary complexes. The identification of such an activity associated with pol III ternary complexes reinforces the prediction that this RNA chain retraction activity may be universal. Several strategies for controlling this activity appear to operate in different systems. Efficient transcript cleavage by pol II is essentially dependent upon the dissociable elongation factor TFIIS, although at least part of the activity may reside within the core polymerase, since retraction, like elongation, is sensitive to α-amanitin (2, 18, 19, 22, 34). The experiments described in this work were carried out with highly purified pol III that was competent to catalyze transcript cleavage in the absence of any added factor. This in itself does not necessarily exclude the action of a dissociable protein. The E. coli elongation factors GreA and GreB remained undetected for some time due to their ability to contaminate even the most highly purified RNA polymerase preparations and their efficient ability to cycle substoichiometrically between ternary complexes (24, 25). Nevertheless, other evidence suggests that this hydrolytic transcript cleavage activity may be intrinsic to pol III, in that ternary complexes retained their ribonuclease activity even after Sarkosyl treatment and Sepharose chromatography had been employed to remove dissociable proteins from DNA-bound polymerase in ternary complexes. Thus, if pol III does employ an elongation factor it is by implication very tightly associated. In view of these considerations, RNA chain retraction by pol III seems much more closely related to the factor-independent activity associated with vaccinia RNA polymerase (31). Here one of the core RNA polymerase subunits, rpo90, has been found to have significant homology with TFIIS and thus it has been proposed that this subunit performs a TFIIS-like function. There are more subunits in yeast pol III/16 at last count; 36) than in either pol II or pol I; conceivably one of these subunits could be related to an elongation factor. Although no statistically significant cross-relationships of amino acid sequence with TFIIS (derived from yeast, human, mouse, and fruit fly), GreA, GreB, or vaccinia virus RNA polymerase rpo90 subunit have been noted in 13 of these pol III subunits (analysis not shown), the amino acid sequence of three candidate subunits with approximate molecular masses of 37, 25, and 11 kDa are not yet known (36).

There is a direct correlation between TFIIS-dependent RNA chain retraction and the ability of TFIIS to overcome blocks to RNA chain elongation that result from both intrinsic DNA sequences constituting arrest sites and obstacles generated by high affinity protein-DNA complexes (2, 8). Although pol III-specific transcription units are almost uniformly short (<200 bp), these sequences contain the binding sites of TFIIC (box A and box B on tRNA [class II] genes) and TFIIB (box C on 5 S (class I) genes), such that TFIIC- and TFIIBA-DNA complexes present potential obstacles to rapid elongation by pol III. The delay to RNA chain elongation contributed by TFIIC bound at its high affinity box B sequence elements has recently been estimated to be ~0.2 s in a crude cell-free system (37), and the delay imposed by bound TFIIC on RNA chain elongation by highly purified pol III was comparably insignificant (28). RNA chain retraction may play a role in facilitating transcription through bound TFIIC (a protein equivalent in size to pol III), and the intrinsic nucleolytic activity present in highly purified pol III ternary complexes is consistent with the rapidity with which purified pol III surmounts the TFIIC-DNA complex obstacle. The rate of retraction observed in this study, in which slightly more than half the active ternary complexes have retracted two nt by 5 s (Fig. 2), is apparently incompatible with chain retraction playing a significant role in limiting the TFIIC-dependent delay of elongation to 0.2 s. However, the presence of NTPs clearly stimulates the rate of retraction by pol III (Figs. 4a and 5), and one might expect that the rate of retraction also has a sequence positional parameter. The appearance of aborted, <16 nt transcription products upon simultaneous addition of MgCl2 and all four NTPs (Fig. 2) suggests that retraction is rapid under these conditions, but this attribute has not yet been examined explicitly. Recently, it has been shown that TFIIS increases the efficiency of transcription by pol II through nucleosomal templates, suggesting that it may play a more general role in transcription than merely relieving elongation arrest at intrinsic termination sites (4). It is therefore tempting to suggest that the intrinsic hydrolytic cleavage activity provides pol III with a similar facility. On the contrary, pol III is incapable of transcribing nucleosomal templates in vivo (5), and thus the hydrolytic activity does not enable pol III to read-through chromatin. All the well defined yeast pol III genes have a requirement for TFIIC in vivo, and it has been suggested that besides assembling TFIIB into a promoter complex TFIIC may also prevent tRNA genes from being assembled into chromatin in vivo (38). Indeed transcriptionally inactive tRNA genes can be incorporated into nucleosomes while active genes are not (39). Other evidence suggests that TFIIC prevents repression of transcription by nucleosomes. The U6 gene can be transcribed in vivo with purified components in the absence of TFIIC (40), but transcription in a crude extract and in vivo absolutely requires the box B element that is the primary determinant of TFIIC binding (42). TFIIC can activate U6 genes after chromatin assembly (43). Thus, pol III assembly factors associated with internal promoter elements may prevent nucleosomes from becoming impediments to RNA chain elongation.

Various small RNA cleavage products have been reported for retraction by pol II. Wang and Hawley (19) observed exclusive production of mononucleotides while others (18, 20, 21) have reported the formation predominantly of dinucleotides and also of some mono-, tri-, and larger oligonucleotides. By comparison, RNA chain retraction by pol III yielded some mononucleotides, but predominantly dinucleotides. The product analysis coupled with the distribution of shortened RNA indicated that the primary pathway for ternary complex retraction from the C31 position on a SUP4 tRNA template is the excision of two pCpU dinucleotides, but that other, undefined retraction pathways occur at a lower frequency. It is likely that the products of this reaction are, to some extent, dependent on the template and the position of the pause, as has been observed for TFIIS-mediated retraction by pol II (20, 21).

Transcript cleavage in pol III ternary complexes appears to occur over a similar time frame to that observed for pol II and the vaccinia virus RNA polymerase. Retraction proceeds much more slowly than does elongation; this presumably ensures that transcript cleavage does not occur until the ternary complex has paused. Retraction by pol III on the tRNA template was less extensive than retraction in other systems. It is not clear whether this is a generalized feature of pol III retraction or a characteristic of the template employed. Indeed when the
reaction was not supplemented with ATP, Mg²⁺-dependent transcripts shorter than 27 nt were not readily detectable even though transcript cleavage could extend backward past C27 to A18 without further loss of label. However, C29 and C27 transcripts did diminish with time implying that retraction past them does occur. Therefore, transcript truncation may occur rapidly and via poorly defined pathways further back than C27. One surprising feature of RNA truncation by pol III was the high proportion of shortened transcripts that were incapable of resuming elongation. This has not been observed in other systems, where the vast majority of cleaved transcripts can chase (22, 25, 31). The formation of inert RNA seems to be linked with transcript release or with a decrease of stability within the ternary complex. The formation of these metastable complexes was observed to occur at particular positions on the template, implying some influence of template sequence on ternary complex stability. This phenomenon may also explain why, during the initial transcription reaction to form C31 complexes, abortive transcripts greater than 10 nt were apparently produced. It would be predicted that complexes stalled at C31 and other positions would retract such that some ternary complexes would re-extend while others would become stagnant and thus not survive column chromatography.

Nucleotides clearly influenced retraction by pol III; this has also been observed for vaccinia virus RNA polymerase (31), and it is conceivable that this may be a feature of factor-independent retraction. Nucleotide precursors were found not to enhance TFIIIS-mediated retraction by pol II (18). The precise molecular basis for the influence of ribonucleotide precursors is not presently clear, but we have noted that a requirement for ATP hydrolysis does not appear likely. Most probably NTP-enhanced retraction results from an allosteric or steric effect of the nucleotide on pol III. The observation that ATP stimulated retraction more than did GDP, and that CTP did not stimulate at all (Fig. 4a), suggests that the presence of the non-cognate nucleotide precursor in the active site may trigger retraction. Alternatively, (relatively slow) misincorporation of the non-cognate nucleotide may initiate (relatively fast) retraction. If pyrophosphorolysis and nucleolytic retraction compete for the re-extended nucleotide within the ternary complex, abor-

ties would retract such that some ternary complexes would re-extend while others would become stagnant and thus not survive column chromatography.

at each end and give the appearance of slow release of the polymerase from a pause site. Kinetic modeling of RNA chain elongation in terms of a series of directly measured, pseudo-first order rate constants fails to generate the observed distribution of chain lengths during elongation by yeast pol III, implying that elongation is mechanistically complex (37). Nucleolytic retraction may be one source of this complexity.

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