SII-facilitated Transcript Cleavage in RNA Polymerase II Complexes Stalled Early after Initiation Occurs in Primarily Dinucleotide Increments*

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RNA polymerase II ternary complex cleaves its nascent transcript in a 3' → 5' direction in the presence of elongation factor SII (Izban, M. G., and Luse, D. S. (1992) Genes & Dev. 6, 1342–1356; Reines, D. (1992) J. Biol. Chem. 267, 3795–3800). We have characterized the cleavage products generated during the transcription process with a variety of stalled RNA polymerase II ternary complexes containing uniformly labeled transcripts. These complexes, which remain elongation competent, had stopped transcription because one nucleoside triphosphate was missing from the reaction mixture. Using a novel assay system, we demonstrate that cleavage occurs in predominately dinucleotide increments, liberating 5'-phosphodinucleotides (pNpNs). In one instance with a particular C20 complex, the first cleavage event was equally partitioned between either a di- or trinucleotide increment with all subsequent truncations occurring by the preferred dinucleotide step. Our data indicate that both the kinetics and the exact increment of SII-facilitated transcript cleavage are influenced by transcript sequence.

Understanding eukaryotic gene expression at the transcriptional level will require a mechanistic appreciation of how RNA polymerase II is regulated during all stages of mRNA synthesis. Examples of regulation during the elongation phase are well documented in both eukaryotic and prokaryotic systems (for reviews see Refs. 1 and 2). A considerable part of our current understanding of transcript elongation by RNA polymerase II has come from in vitro transcription experiments, using ternary complexes initiated either at promoters or on 3'-ts-tailed templates (see, for instance, Refs. 7–9 and references therein). RNA polymerase II ternary complexes which do not contain elongation stimulatory factors (core complexes) transcribe at approximately 300 nucleotides (nt)/min in vitro (9), well below the estimated in vivo elongation rate of 1200–1500 nt/min (10). A significant portion of core complexes also become arrested in elongation while transcribing through regions of DNA (originally termed intrinsic termination sites) thought to influence gene expression in vitro (5, 7, 9, 11–15). For clarity, these sites will be referred to as intrinsic arrest sites since promoter-initiated RNA polymerase II complexes trapped at these sites do not actually terminate transcription (4, 7, 9, 13). Intrinsic arrest sites have been found in the 5’-portion of a number of viral and cellular genes (reviewed in Refs. 1 and 2).

Two classes of elongation factors which interact transiently with the RNA polymerase II ternary complex and influence the processivity and efficiency of transcription in vitro have been discovered. One group of factors, exemplified by HeLa cell TFIIF, increases the rate at which transcript elongation occurs (4, 9, 16). TFIIF alone is capable of stimulating transcription to physiological rates in vitro on naked DNA but not on chromatin templates (9). However, TFIIF does not abolish the block to elongation encountered at intrinsic arrest sites (4, 9). The second category of elongation factors includes homologs of SII (17–20) and SII-related proteins found in mouse (21). Under near physiological conditions, using either dC-tailed or promoter-initiated ternary complexes, SII enables core polymerase to read efficiently through intrinsic arrest sites. This effect is observed regardless of whether the SII is added prior to or after the block to elongation has occurred (4, 5, 7, 9, 11, 13, 15, 22). Moreover, SII does not prevent transcriptional arrest. Elongation is temporarily blocked during transcription through arrest sites even in the presence of SII; the resumption of transcript elongation occurs some time later (9). The effects of SII and TFIIF on elongation are additive; thus, these factors may interact at different sites or upon different configurations of the ternary complex (9).

The mechanism by which SII alleviates the block to elongation has only recently been elucidated. Remarkably, arrested RNA polymerase II ternary complexes cleave their nascent transcripts in a 3' → 5' direction in the presence of elongation factor SII (6, 15). Most importantly, cleavage of the nascent RNA is required for the resumption of transcription from an arrest site (Ref. 23, and accompanying article, Ref. 33). The SII-facilitated nuclease activity appears to be a general property of polymerase ternary complexes, as a variety of artificially stalled complexes generated from two different promoters exhibit this activity (6, 15). Characterization of the truncation reactions using ternary complexes stalled at defined sites along the template has shown that even after extensive transcript shortening, polymerase exactly recreates the RNA fragment that was removed when RNA synthesis resumes from the newly-created 3'-end (15). Thus, the transcript and catalytic site of the polymerase remain in register with the underlying DNA during transcript truncation. RNA polymerase II itself must participate in the SII-facilitated

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The abbreviations used are: nt, nucleotide; pNpNs, 5'-phosphodinucleotides; SII, recombinant human SII; TEAA, triethylammonium acetate; pNpNpNs, 5'-phosphotrinucleotides; Py, pyrimidine; Pu, purine; NTP, nucleoside triphosphate.
cleavage reaction since the nuclease activity is inhibited by α-amanitin (6, 15). *Escherichia coli* RNA polymerase also exhibits a nuclease activity in the presence of the bacterial GreA protein (24, 25). Both highly purified mammalian ternary complexes (6, 15) and *E. coli* RNA polymerase reconstituted from purified subunits (24) exhibit a low level of cleavage activity in the absence of added factors, suggesting that the nuclease activity may be an inherent property of the RNA polymerase ternary complex.

To characterize the truncation reaction further we have identified the products generated by the SII-facilitated nuclease activity. We used a variety of ternary complexes stalled early after initiation (this study) and ternary complexes either stalled or blocked in elongation within regions similar to intrinsic arrest sites (33). In this report we show that cleavage within complexes stalled early in the elongation process usually occurs in dinucleotide increments, liberating 5'-phosphodinucleotides (pNpNPs). With one particular complex we demonstrate that the first cleavage cycle occurred in either a di- or trinucleotide increment with subsequent cycles liberating only pNpNPs.

### Materials and Methods

All unlabeled ribonucleoside triphosphates (standard purity and ultrapure, fast protein liquid chromatography purified) were purchased from Pharmacia LKB Biotechnology Inc. [α-32P]CTP and [γ-32P]ATP (3000 Ci/mmol) were obtained from Du Pont-New England Nuclear. Sodium pyrophosphate was purchased from Sigma. Dinucleotides and trinucleotides were either purchased from Sigma or synthesized (in the case of GpCppG and GpCpG) on an Applied Biosystems synthesizer. Bio-Gel A-1.5m was obtained from Bio-Rad.

**Plasmids**—All plasmids except pMB5-ML have been described elsewhere (15). Basically, the pML-based plasmids contain different variations of the adenosine major late promoter: pMB5-ML contains the major late promoter from -50 to -12 fused to a modified version of mouse β-globin initial transcription sequence from +14 to +21; the initial transcribed sequence, beginning at +1, is 5'ACUCUCUC-UCCCCCGCGGCCGCA...

**Elongation Factors**—Elongation factor SII was purified to apparent homogeneity (as described previously; Ref. 8) from calf thymus; this preparation was the gift of R. Weinmann (Wistar Institute). The concentration of SII was 0.077 mg/ml. Purified recombinant human SII (rSII) was a gift from R. Landick (Washington University, St. Louis, Department of Biology) and was purified as described by Yoo et al. (17). The concentration of rSII as determined by the Bradford assay was 0.5 mg/ml.

**Assembly and Purification of Paused Elongation Complexes**—A detailed protocol has been described elsewhere for preinitiation complex assembly (14). Briefly, template DNA was incubated in HeLa cell nuclear extract to allow RNA polymerase and initiation factors to assemble on the promoter, followed by a gel filtration step. Defined elongation complexes labeled only in the first 10–12 nt were prepared from preinitiation complexes as described (15). When uniformly labeled prepaused ternary complexes were generated, initiation reactions contained 10 μM [α-32P]CTP or [γ-32P]UTP at 800 Ci/mmol. Typically, 40 μl of labeling nucleotide was dried under vacuum and resuspended in 5.5 μl of 10 X initiation nucleotides (20 mM ATP, 100 μM dATP, and 100 μM of either UTP or CTP depending on the labeling base). After the addition of 50 μl of preinitiation complex, the reaction was incubated at 25 °C for 10 min prior to purification of stalled ternary complexes by a previously described procedure we termed Sarkosyl rinsing (14). Basically, stalled complexes were incubated for an additional 5 min after the addition of Sarkosyl to 1% followed by column chromatography through Bio-Gel A-1.5m (Bio-Rad Laboratories); typically, 10 μM of Tris-Cl (10 mM NaCl, 10 mM MgCl2, 62 mM KCl, 0.5 mM EDTA, and 1 mM dithiothreitol) or MEMM devoid of the phosphatase inhibitor β-glycerophosphate as running buffer. This procedure removes the nucleotides and Mg2+ ions required for initiation as well as the Sarkosyl and proteins remaining from Sarkosyl. SII-facilitated truncation reactions were performed as described (15) and typically contained either calf thymus SII (2.5 μg/ml) or human SII (3.5 μg/ml). All reactions were terminated by the addition of stop buffer, typically 70 μl (20 mM EDTA, pH 8.0) so that the final volume was 100 μl. After proteinase K digestion (200 μg/ml) for 0.5–2.0 h, samples were sequentially phenol-chloroform and chloroform extracted prior to lyophilization. Reactions were resuspended in 6–8 μl of H2O. When phosphatase treatment was performed prior to electrophoresis, samples were resuspended in 10 μl of H2O and incubated at 35 °C for 15 min after the addition of 1 unit calf intestinal phosphatase (Boehringer Mannheim). RNAs were purified by sequential phenol-chloroform and chloroform extractions, concentrated by lyophilization, and resuspended in 6–8 μl of H2O. An equal volume of formamide dye mixture was added to 2 μl of reaction mixture prior to gel electrophoresis at 10 watts, using constant power, on short (12.5 cm) or long (23.5 cm) 28% acrylamide (25:3 acrylamide:bisacrylamide) sequencing gels with TBE (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.0) as running buffer. The bromophenol blue dye marker was run to 12.5 cm on both short and long gels except for the long gel shown in Fig. 4 where it was run to 23 cm.

**Descending Cleavage Products Isolated from Gels**—The dinucleotide cleavage products were resolved by 28% polyacrylamide gel electrophoresis and eluted from the gel slices in 100 μl of 100 mM triethylammonium acetate, pH 7.4 (TEAA), by the crush-soak method. 400 μl of H2O was added to bring the TEAA concentration to 20 mM, and the sample was loaded onto 15-cm DE52 (Whatman) ion exchange column (made in a 1-ml syringe) equilibrated in 20 mM TEAA. The column was washed with 1 ml of 20 mM TEAA prior to elution of the pNPnPNs with three 200-μl volumes of 1 M TEAA. The volatile TEAA buffer was removed by drying under vacuum. Residual TEAA was removed by additional drying cycles after the addition of 100 μl of H2O. The final material was resuspended in 0.1 × TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

**Preparation of Di- and Trinucleotide Standards**—The dinucleotide CpG was generated by digesting GpCpG with RNase A (Sigma). Radiolabeled pNPnP and pNPpNPs were produced in 30-μl reactions containing 5 units of polynucleotide kinase (Pharmacia), 25 μmol of di- or trinucleotide, 0.06 μM [γ-32P]ATP, 70 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 5 mM dithiothreitol. Reactions were incubated for 30 min at 37 °C followed by the addition of 1 μl of 0.2 mM ATP and an additional 30-min incubation. In some reactions not all of the radiolabeled ATP was consumed.

**Results**

**Rationale and Assay System**—We have previously shown that the presence of elongation factor SII, ternary complexes exhibit nuclease activity which shortens their nascent transcripts in a 3' → 5' direction. We performed a detailed analysis of truncation kinetics using a variety of ternary complexes stalled early after initiation on either the adenosine major late or mouse β-globin transcription units. The lengths of the shortened transcripts strongly suggested, but did not prove, that truncation occurs in primarily dinucleotide increments (15). To resolve this issue we re-examined SII-facilitated cleavage with a variety of these stalled complexes that contained uniformly labeled transcripts, which allowed us to characterize the cleavage products directly. Since polymerization effectively competes with the cleavage reaction in the case of stalled complexes, we purified the elongation complexes (see "Materials and Methods") to remove the nucleotides. After purification, the majority of stalled ternary complexes remained fully functional; they either resumed transcription elongation, if supplemented with Mg2+ and NTPs, or they began transcript truncation upon addition of Mg2+ and elongation factor SII (see Fig. 5A, lanes 2 and 3–7).

We initially attempted to characterize the truncation products by employing polyethyleneimine electrophoresis. However, this method proved unsuitable because the cleavage products (pNPnPNs) were poorly resolved and often comigrated with nucleoside monophosphates. We found that pNPnPNs were resolvable on 28% sequencing gels (Fig. 1A; for example, see lanes 4–7). In general the mobilities were pYPpPy > pPypPu/pPupPu > pPupPy. However, dinucleotides that had identical base composition but different sequence (for instance, pGPpA and pAPGp) were not resolvable (lanes 4–6).
FIG. 1. The cleavage products released by SII-facilitated truncation are primarily pNpNs. U20 complex uniformly labeled with either CTP (U20-C; panels A and C) or UTP (U20-U; panel B) was incubated for 30 (SII) or 15 (tSII) min as indicated. Portions of the
The 28% gels also resolved pNpNs from the two 32P-nucleotides used to label the transcript (Fig. 1, lane A1, CTP, and lane B3, UTP). Furthermore, all three phosphorylated forms of both C (Fig. 1A, lane 1) and U (Fig. 1B, lane 3) nucleotides could be resolved with salt unavoidably introduced via the truncation reaction buffer, however, decreased the resolution between the tri- and monophosphate forms of both nucleotides. We also found that salt slightly affected the mobility of the pNpNs and pNpNpNs (shown in Fig. 3, lanes 1 and 2). Since the salts similarly affected the mobility of the bromphenol blue tracking dye (data not shown), the position of the dye in the lanes containing standards and reaction mixtures was used, when necessary, as a point of reference to determine relative Rf of the various cleavage products.

SII-facilitated Truncation of Nascent Transcript within U20 Complex Occurs Primarily in Dinucleotide Increments—We first sought to determine the SII-facilitated cleavage products using U20-C (Fig. 1A) and U20-U (Fig. 1B) complexes. By convention, stalled complexes are named according to the base at the 3’-end and the length of the transcript. Terminy complexes containing uniformly labeled transcripts are further identified by appending the labeling base to the complex designation. Unfortunately, trace amounts of unincorporated radiolabeled nucleotide, in predominantly the tri- and diphosphate forms, unavoidably contaminated our purified ternary complexes (Fig. 1, lanes A2 and B2). As expected, the radiolabeled phosphate was removed from these nucleotides by calf intestine phosphatase treatment (Fig. 1, lanes A3 and B1). Since the complexes generated in this study were initiated by dinucleotide priming with ApC, all transcripts lacked a 5’-triphosphate. As expected, phosphatase treatment did not alter the mobility of the purified 20-nt transcript (compare Fig. 1, lanes A2 to A3 and B2 to B1).

The sequence of the transcript within U20 complex is 5’-ApCpUpC pUpC pUpC pCpC pUpU pCpG pCpU pUpU-3’. The dinucleotide spacing indicates the predicted SII-facilitated cleavage products; the locations of radiolabeled phosphates within the complexes are underlined (for U20-C) or bold-faced (for U20-U). Consistent with our previous study, incubation of U20 complex in the presence of 2.5 mg/ml bovine SII for 30 min generated a heterogeneous population of truncated transcripts extending down to 4 nt (Fig. 1, lanes A8 and B5). U20-C complex liberated pCpG, either pCpU or pUpC, and pCpC (Fig. 1A, lane 8) while U20-U complex generated pUpU and either pCpU or pUpC (Fig. 1B, lane 5). The pUpU standard runs between pCpUpU and pCpA/pApC; the relative mobilities of pPpPy dinucleotides in this gel system are pCpG > pCpU/pUpC > pPpUpU (see Fig. 2, lane 1; some data not shown). We could not determine by inspection of the radiolabeled pNpNs whether both pCpU and pUpC were produced. Note, however, that phosphatase treatment would remove the radiofiled phosphate from pCpU but not pUpC in the U20-C reaction and from pUpC but not pCpU in the U20-U reaction. Thus, we could unequivocally demonstrate that pCpU and pUpC were generated in both reactions since we observed the predicted reduction in radiolabel associated with UpC (Fig. 1A, lane 10) and CpU (Fig. 1B, lane 4). Also as expected, the amount of radiolabel within both CpC (U20-C, Fig. 1A, lane 10) and UpU (U20-U, Fig. 1B, lane 4) was half that found within pCpC (U20-C, Fig. 1A, lane 8) and pUpU (U20-U, Fig. 1B, lane 5). Because of the heterogeneity in the extent of transcript truncation it is difficult to predict exactly the ratio of liberated pNpNs in any given reaction. The relative abundance of the liberated products, however, was always consistent with the observed extent of truncation.

We also purified the pNpNs generated from U20-C complex by ion exchange chromatography using a volatile running buffer (see "Materials and Methods"). After removal of the salt by lyophilization, this sample was rerun on a 28% sequencing gel to demonstrate comigration with the appropriate markers (Fig. 1C, lane 7). A portion of this sample was phosphatase treated and subjected to polyethyleneimine thin layer chromatography developed in 0.16 M LiCl. As expected we observed radiolabeled UpC, CpC, and inorganic phosphate (data not shown).

The amount of radiolabel within the 20-nt transcript was essentially identical to that found within the truncated transcripts and released dinucleotides, with approximately 60% of the radiolabel found within the released pNpNs shown in Fig. 1A, lane 8. Thus, the strongly preferred increment of SII-facilitated cleavage in stalled U20 complex is by dinucleotides. The total amount of CTP, CDP, and CMP, however, increased by 5% relative to the radiolabel within the 20-nt transcript during the truncation reaction. This increase may simply reflect variation in recovery during purification as we did not observe this increase with other C-labeled complexes; also, the amount of radiolabeled UTP, UDP, and UMP present in the U20-U reaction was essentially identical before (Fig. 1B, lane 2) or after (Fig. 1B, lane 5) truncation. The low but detectable amount of CMP produced (Fig. 1A, lane 8) may have been generated by the removal of phosphate from the contaminating nucleoside di- and triphosphates. The purified ternary complexes contain a low level of phosphatase activity since NpNs were generated at low levels after the 30-min "back-up" reaction (Fig. 1, lanes A8 and B5). However, we cannot rule out the possibility that an SII-facilitated mononucleoside cleavage occasionally occurred. In fact, it is reasonable to suppose that ternary complexes may produce nucleoside monophosphates at some low frequency in the presence of SII since liberation of nucleoside monophosphates does appear to occur in SII-facilitated truncation reactions supplemented with NH4 or if Mn2+ is used as the divalent cation (Ref. 15 and data not shown).

The bovine SII we use to facilitate the cleavage reaction is of extremely high purity (8). It is formally possible that a contaminating activity(s) in this preparation facilitated the truncation reaction. This seems very unlikely, however, as bacterially expressed and purified recombinant human SII exhibited the same activity (Fig. 1A, lane 11; see also below). Note that the amount of U20-C complex used in this reaction was approximately one-fourth the amount of U20-C complex displayed in all other lanes. We also observed low levels of large polynucleotide cleavage products (like pNpNs, their mobilities were also altered after phosphatase treatment) in reactions performed with either calf thymus (Fig. 1, lanes A8 and B5) or recombinant human SII (Fig. 1A, lane 11). This reaction will be discussed in more detail below (see Fig. 4) and in the accompanying paper (33).
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We demonstrated that SII-facilitated cleavage occurs in dinucleotide increments with these complexes using either a pCpG or a 30-min (lane 4) reaction. Approximately 70% of the radiolabel was found within the liberated pNpNs after the 30-min reaction. The relative abundance of these products at both time points was consistent with the extent of truncation and the predicted cleavage products. For instance, comparison of the pNpNs liberated in the 5- and 30-min reactions shows that the ratio of pCpG:pCpU:pUpC increased with time. Note that more than half of the complexes still retained 11–13 nt RNAs in the 5-min reaction. The dinuclease pCpG can only be produced during the third cleavage cycle of C15 complex (C11 → C9) and the fourth (C12 → C10) and fifth (C10 → U8) cleavage cycles of U18 complex. We also observed very low levels of pCpG relative to the amount of other pNpNs; this value was 3% (lane 4) compared to 11.5% generated with U20-C complex (Fig. 1A, lane 8). Furthermore, the amount of pCpG was essentially identical in reactions performed for either 5 (Fig. 2, lane 3) or 30 (lane 4) min. These observations are consistent with the facts that pCpG can only be generated by U18 complex during the second cleavage cycle (which was complete by 5 min) and that C15/U18 complex contained 25% U18 complex. This gel also clearly shows the effect salt can have on the migration of pNpNs (compare the pCpC and pCpG markers in Fig. 2, lane 1, with the liberated products in lanes 3 and 4). The mobilities of the phosphatase-generated CpC and UpC (Fig. 2, lane 5, and see Fig. 4) verify the identity of the liberated products. Finally, note the mobility of pUpU relative to pCpC (Fig. 2, lane 1; this was not shown in Fig. 1).

We also determined the cleavage products generated from U35-U complex (Fig. 3, starting material shown in lane 7). The sequence of the transcript within U35-U complex, where the radiolabeled phosphates are bold-faced and spaced indicates the predicted dinucleotide cleavage products, is: 5'-A pCpC pCpU pCpC pCpC pCpC pUpG pUpU pUpG pUpG pUpG pUpG pUpG pUpG pUpU. A 5-min truncation reaction (lane 3) generated the exact pattern of shortened transcripts observed in our previous report (see Fig. 5, lanes 7 and 9, of Ref. 15). The metastable C31 complex predominates with trace levels of complexes containing 29, 27, 25, 23, 21, 17, 12, and 10 nt transcripts. This pattern was confirmed by running a portion of the reaction on a 15% sequencing gel (data not shown here; see also Fig. 4, lane 1).

As expected, the cleavage products pCpU and presumably pUpG were detected after the 5-min back-up reaction. We did not have an appropriate marker for pUpG, but the pNpN we presume to be pUpG ran slightly above pCpG, consistent with the general trend we observed for migration of 5'-pNpNs, i.e. pPyPy > pPyPu/pPyPy > pPyPu. Truncation for 30 min (Fig. 3, lane 5) generated additional pCpU, pUpG, and pUpC as expected, based on an exclusively dinucleotide cleavage increment. However, pUpU was also generated. Its identity was confirmed by the appearance of UpU after phoshatase treatment (Fig. 3, lane 6 and see Fig. 4, lane 3). This last observation indicated that a cleavage(s) in other than the favored dinucleotide increment is possible.

We have performed a 5–30-min time course to carefully monitor the retreat of U35 complex and found that cleavage by dinucleotide increments is preferred until C17 complex is generated (data not shown). To liberate UpU, C17 complex may cleave its nascent transcript in either a single or trinucleotide increment followed by resumption of the favored dinucleotide cleavage. Using U35-C we indeed observed the trinucleotide product pCpGpC; however, it was not produced stoichiometrically by C17 complex (data not shown). This is consistent with the low level of UpU relative to CpG generated after a 30-min back-up reaction (compare CpG and UpU in the phosphatase-treated samples, Fig. 3, lane 6 and Fig. 4,
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Fig. 3. Products liberated during the cleavage reaction with U35 complex uniformly labeled with UTP (U35-U). Truncation reactions were performed for the times indicated. Markers were run in lanes 2 and 4. The markers in lane 2 were also mixed with an equivalent amount of reaction product to that shown in lane 6 and the mixture was run in lane 1, to illustrate the effect of salt on the mobility of the markers. Products were resolved as in Fig. 1. Transcription lengths and markers are indicated at left. CIP, calf intestinal phosphatase.

lane 3). We propose that pUpU and pCpGpC were generated from U35-U by the following truncation scheme: 5'-ApC pUpC pUpC pUpC pUpC pCpC pUpG pUpG pUpG pCpG pUpG pCpG pUpG pCpG pUpG pCpG pUpG; this progression is presumably superimposed on the dinucleotide cleavage pattern indicated above. We have also demonstrated (see Fig. 5) that a different ternary complex can truncate its transcript in mutually exclusive di- and trinucleotide increments.

Transcript Cleavage in Stalled Complexes Can Also Occur to a Limited Extent in Larger Increments—The data presented in Figs. 1–3 clearly demonstrate the preference for the production of dinucleotides during SII-facilitated transcript cleavage in stalled complexes. However, based on alteration in mobility of some longer RNAs after phosphatase treatment, we also observed low levels of large cleavage products with U20 and U35 complexes. While pNPNS and pNPNPNS were resolved from the shortest labeled truncated transcript (4 nt), the larger cleavage products were not well resolved from transcripts of the same length. To increase the resolution between shorted transcripts and large cleavage products we ran a portion of the reactions displayed in Figs. 1–3 on the longer 28% gel system, allowing the bromphenol blue dye to run to the bottom (Fig. 4; U20-C complex, lanes 8 and 9; U20-U complex, lanes 4 and 5; C15/U18 complex, lanes 10–12; and U35-U complex, lanes 1–3). Transcripts from a 15-min back-up reaction utilizing rSII and U20-C complex (a different preparation than the one displayed in Fig. 1A) either before (Fig. 4, lane 6) or after phosphatase treatment (Fig. 4, lane 7) are also shown.

Interestingly, we saw no evidence for a large cleavage increment with C15/U18 complex (compare Fig. 4, lanes 11 and 12). The mobilities of the shortened transcripts were unaffected by phosphatase treatment and the shortened tran-
FIG. 5. SII-facilitated and pyrophosphorylytic transcript cleavage using C20 complex generated on the pMB5-ML template. A, C20 complex was supplemented with Mg²⁺ and SII and incubated for the times indicated. Reactions were chased by adding NTPs to 1 mM and incubating for 2 (lane 2) or 0.25 (lane 8) min. Transcripts were resolved as described in the legend to Fig. 1 except that a short (12.5 cm) gel was used. Transcript lengths are indicated at left. B, C20 complexes uniformly labeled with CTP (C20-C) were used in truncation reactions for the times indicated. The reaction in lane 5 was treated with calf intestinal phosphatase prior to electrophoresis; note that this phosphatase reaction did not go to completion. Markers were run in the indicated middle lanes; they are identified, along with selected transcripts, at right. The boxes indicate contaminants of unknown composition in the markers lane. C, C20 complex (lanes 3–14) was supplemented with Mg²⁺ and 100 μM pyrophosphate and incubated for the times indicated. Lane 14 was subsequently chased for 30 s after the addition of NTPs to 1 mM. Complex C20 was incubated for 10 min with Mg²⁺ (lane 1) or for 5 min with Mg²⁺ and SII (lane 2). Transcripts were resolved as described in A. Transcript lengths are indicated at the right and left.
scripts comigrated with transcripts generated by stalling ternary complexes using reactions containing limiting NTPs (data not shown). Since the templates used to generate C15/U18, U20, and U35 complexes have identical initial transcribed sequences through +18, the shortened transcripts derived from C15/U18 served as mobility markers to distinguish shortened transcripts from truncation products in the U20 and U35 reactions.

As noted earlier, the large cleavage products generated by U20 complex contained 5'-terminal phosphates since their mobilities were decreased after phosphatase treatment (compare the untreated and phosphatase-treated reactions, Fig. 4, lanes 4–9). For example, the product found just below the 8-nt transcript derived from U8 complex disappeared upon phosphatase treatment with the concomitant appearance of a band of similar intensity just above 8 nt. Note that the larger fragments undergo smaller changes in their charge to mass ratios than do pNpNs after phosphatase treatment and consequently exhibit less difference in migration. After phosphatase treatment the large cleavage products are still distinguishable from shortened transcripts of comparable lengths because of differences in base composition (Fig. 4, lanes 4–9). While these subtle differences in mobilities make it impossible to assign exact lengths, the relative mobilities suggest that the cleavage reaction liberated low levels of 6–11-nt fragments. Based on the apparent absence of the large cleavage increment with C15/U18 complex, we strongly suspect that U20 complex generated all the large cleavage products (in the accompanying report (33) we demonstrate that a single complex is capable of generating a heterogeneous population of cleavage products). Thus, we would expect that the large cleavage products would contain the 3'-end of the U20 transcript. Indeed, the relative intensities of the large cleavage products generated with either U20-C or U20-U correlate with the U:C base ratio in the distal portion of the transcript. For instance, if the 8-nt cleavage product was produced by U20-U complex then its sequence would be 5'-pUpUpCpGpCpUpUpU and it would contain 2.5 times the radiolabel as the identical product generated with U20-C complex. Transcripts shortened to 6 and 10 nt can be used as a reference since the ratio of radiolabeled U and C residues within these RNAs is identical. It is also noteworthy to mention that in our initial characterization of the back-up reaction we failed to observe the large cleavage products with U20 complexes containing transcripts labeled only within the first 10–12 nucleotides.

Lastly, we also observed the same set of large cleavage products in reactions performed with the purified recombinant SII (Fig. 4, lanes 6 and 7) with the exception of the cleavage product that comigrated with the 10-nt transcript prior to phosphatase treatment. This subtle variation could either reflect genuine differences between the human and bovine proteins or simply be the consequence of a more active rSII preparation. Note that a 15-min reaction with rSII resulted in more extensive transcript truncation than a 90-min reaction with a similar concentration of bovine SII (compare the 4- and 10-nt transcripts in Fig. 4, lanes 5, 7, and 9).

U35-U complex also generated low but significant levels of larger cleavage products ranging from 8 to 12 nt in length (compare Fig. 4, lanes 2 and 3). The most abundant of these products must differ in sequence from the most prominent large RNA released by U20 complex, since the fragment released from U35 comigrates with the transcript associated with C9 complex and is shifted to just below the C10 transcript after phosphatase treatment. We are reasonably confident that this large cleavage product was generated after the initial dinucleotide cleavages of U35 and G33 complexes because it was under represented in the 5-min back-up reaction that contained predominantly C31 complex (Fig. 4, lane 1, and additional data not shown). In summary, the data in Fig. 4 demonstrate that transcript truncation within stalled complexes can also occur, albeit infrequently, in large increments. However, a critical transcript length may be required for the large cleavage increment as large truncation products were generated by U20 and U35 complexes but not by C15/U18 complex.

**Transcript and/or Underlying DNA Sequence Influence the Cleavage Reaction**—We previously reported that the cleavage kinetics and the pattern of shortened transcripts are completely different with complexes generated from the mouse β-globin and adenovirus major late promoters (15). Furthermore, the first SII-facilitated cleavage cycle of the β-globin C21 complex appeared to favor a trinucleotide increment. To investigate further the influence of transcript sequence on the cleavage reaction we constructed a template containing the adenovirus promoter and initial transcribed region to +12 followed by the early transcribed region from +14 to +21 of the mouse β-globin promoter. We have designated this template pMB5-ML. The sequence of the transcript within C20 complex (the β-globin derived region is underlined) is: 5'-ACUCUCUCCCCGCGGCCGC.

Consistent with the results obtained with all other stalled complexes we have tested, C20 complex made on pMB5-ML (Fig. 5A, lane 1) resumed transcript elongation when supplemented with Mg2+ and NTPs (Fig. 5A, lane 2). Complexes which had truncated their transcripts to as short as 9 nt remained elongation competent as they chased upon NTP addition (Fig. 5A, lane 8). A time course of the cleavage reaction (Fig. 5A, lanes 3–7) revealed two very interesting results. First, the kinetics and transcript patterns generated during truncation through the β-globin sequence were essentially the same as we previously reported with the β-globin promoter C21 complex (see Ref. 15, Fig. 6). As truncation proceeded through the adenovirus sequence (Fig. 5A, lane 7) the pattern was similar to that observed with C15/U18 (Fig. 2, lane 3), as opposed to the pattern generated during truncation with the entirely mouse globin-derived C21 complex (see Ref. 15, Fig. 6). Second, the initial cleavage reaction appeared to occur with roughly equal probability in either a di- or trinucleotide increment (Fig. 5A, lane 3); the 18- and 16-nt transcripts were presumably generated by two cycles of dinucleotide cleavage (see below).

To determine the cleavage products we performed the truncation reaction with uniformly labeled C20-C complex generated on pMB5-ML (Fig. 5B, lane 1) and displayed the reactions on a long 28% sequencing gel. If the first cleavage liberated either a di- or a trinucleotide, followed by cleavage only in dinucleotide increments, then the anticipated cleavage products would be (the radiolabeled phosphates are bold-faced): 1) 5'-pCpU pCpU pCpU pCpU pUpC pCpC pCpG pGpC pCpG pGpC, 2) 5'-pCpU pCpU pCpU pCpU pCpC pCpG pGpC pCpGpC, and finally pUpC and pCpU dinucleotides. This explains why truncation through the major late sequence mimics the retreat pattern produced by C15/U18 complex. Note also that we observed very little large cleavage increment with C20 complex. Only the faint band that migrates just below 9 nt disappeared upon phosphatase treatment (lane 5). Thus,
other considerations (such as a run of U residues at the 3'-end of the transcript within U20 complex) may enhance the probability of a large cleavage increment (see accompanying paper, Ref. 35).

Pyrophosphorolysis by RNA Polymerase II—For comparison with SII-facilitated transcript cleavage, we also investigated pyrophosphorolysis by RNA polymerase II (Fig. 5C, lanes 4-13). Pyrophosphorolysis is simply the reverse of the polymerization reaction driven by the addition of excess PPi (26). Thus, in the presence of PPi, ternary complexes should shorten their transcripts in single nucleotide increments, consuming PPi and liberating in triphosphate form the base located at the 3'-end of the transcript. The time course in Fig. 5C shows that pyrophosphorolysis is a relatively slow reaction (relative to SII-facilitated cleavage) even at 100 μM pyrophosphate (lanes 4-13). Interestingly, metastable ternary complexes containing 16- and 13-nt RNAs formed during pyrophosphorolysis; note that different metastable complexes are generated during SII-facilitated cleavage of the same transcript (compare Fig. 5C, lane 2 with lanes 3-13). Transcripts were retained in elongation competent ternary complex during pyrophosphorolysis as the addition of excess NTPs after a 20-min reaction efficiently chased the shorter transcripts (lane 14). Pyrophosphorolysis by uniformly labeled complexes generated no detectable dinucleotide cleavage products. Finally, note that transcript cleavage occurs with C20 complex upon extended (10 min) incubation with Mg2+ alone (lane 1). This agrees with our previous observations using a number of different stalled complexes (15). Thus, either an SII or SII-like activity contaminates our purified complexes or SII strongly facilitates a nuclease activity inherent to ternary complexes.

**DISCUSSION**

A more complete understanding of the modulation of eukaryotic gene expression during the elongation phase of transcription will require a mechanistic appreciation of the influence of elongation factors on RNA polymerase II. In vitro, SII works primarily to increase processivity. It is required for efficient elongation through regions of DNA (intrinsic arrest sites) that produce blocks to elongation at an intrinsic arrest site (23, 33). However, this transcript cleavage activity is a general property of ternary complexes since a variety of elongation competent, artificially stalled complexes efficiently truncate nascent transcript in the presence of SII (Refs. 6, 15, and this study). In this work we demonstrate that ternary complexes acquire comparable nuclease activity in the presence of either purified bovine or recombinant human SII. Using a variety of stalled complexes we show that a dinucleotide cleavage increment is preferred, resulting in the liberation of pNpNs. We also establish that transcript sequence may influence the cleavage reaction, exemplified by particular complexes that exhibit mutually exclusive di- or trinucleotide cleavage increments (Fig. 5).

**Does Sequence Influence the Specificity of the Cleavage Reaction?**—We have previously shown with a variety of ternary complexes stalled early in elongation that metastable ternary complexes form during the SII-facilitated truncation reaction when the residual nascent transcript approaches 10-12 nt in length. We feel that this reflects a fundamental transition point in ternary complex stability as transcripts which were truncated further are not elongated (for a complete discussion see Ref. 15). In many instances, we also observed striking variations in the kinetics of truncation with particular ternary complexes containing fairly long transcripts. Unlike the 9-12 nt transition, however, further truncation by these metastable complexes does not affect the stability of the ternary complex (see, for example, Fig. 5A). We had previously suggested that the variable stability of these complexes might reflect attributes of the translocation process (see "Discussion" in Ref. 15). It now seems likely that the formation of these metastable complexes is additionally, and perhaps predominantly, influenced by the effect of transcript sequence on the kinetics of truncation.

Although our data set is small, there are some general features of transcript sequence associated with reduced kinetics of truncation. In every case, the formation of metastable complexes occurs just prior to G-rich regions in the transcript. For instance, truncation by U35 and C20 complexes generated metastable complexes at C31 and C17, respectively. The transcripts of both complexes contain consecutive G residues near the next cleavage site; the 3'-ends of the transcripts are shorten through these regions in the favored dinucleotide cleavage increment. We note that transcript cleavage by the C17-derived G15 complex was very fast as negligible levels of the 15-nt transcript were observed (Fig. 5). This suggests that metastable complexes are not simply the result of enhanced heteroduplex stability within GC-rich regions. On the other hand, cleavage through the pyrimidine-rich U20 transcript showed very little variation in kinetics until, as indicated above, the complex contained 9-12 nt transcripts (see Ref. 15). Clearly a detailed analysis of truncation kinetics through a variety of transcript sequences is warranted. More compelling evidence for the influence of transcript sequence alone on the nuclease activity was obtained with C20 complex itself a metastable complex, which produced both di- and trinucleotide cleavage products in roughly equimolar amounts (Fig. 5B). Similarly, the metastable C17 complex observed in truncation reactions with U35 complex also generated di- and trinucleotide cleavage products (see Fig. 4, and additional data not shown). Remarkably, both C17 and C20 complexes liberated the same trinucleotide, pCpGpC. While C20 complex shares some underlying DNA sequence similarity to C17 complex (recall C20 complex was generated with a major late/β-globin fusion construct) we had originally shown that the β-globin promoter-derived C21 complex exhibited the trinucleotide increment (15). The only apparent sequence similarity within the major late C17 and β-globin C21 complexes is the sequence (5'-CpGpC-OH) of the transcript 3'-end. The influence of transcript sequence on the cleavage reaction is further supported by the truncation pattern observed with a major late/β-globin initial transcribed region fusion construct (Fig. 5A). The truncation kinetics and transcript patterns generated through the β-globin and major late portions of the

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transcript mimic the transcript patterns produced by truncation through the identical regions of the transcript within β-globin C21 complex (15) and major late C15/U18 complex.

Prokaryotic RNA polymerase ternary complexes also exhibit nuclease activity in the presence of the bacterial protein GreA. This truncation reaction generates di-, tri-, and decanucleotide cleavage products containing 5'-terminal phosphates (24, 25). We find it interesting that the trinucleotide product (pCpAP) reported in the study by Surratt et al. (25) is very similar to the pCpGpC cleavage product we observed. Surratt et al. also showed that two particular complexes truncated their nascent transcripts in a large cleavage increment. However, the release of larger fragments was at best a minor reaction during SII-facilitated cleavage with the stalled complexes tested in this study. In the accompanying article (33) we demonstrate circumstances in which the large cleavage increment can dominate the reaction.

What Constitutes the Nuclease Activity?—SII (for RNA polymerase II) or GreA (for E. coli RNA polymerase) facilitates transcript cleavage only when transcript is within ternary complex (6, 15, 24), a process that requires a divalent cation and is inhibited, in the case of RNA polymerase II, by α-amanitin (6, 15). Thus, RNA polymerase ternary complex GreA is likely to contain at least a portion of the active site. RNA polymerase II was predicted to possess an RNase-like domain based on sequence similarities between its second largest subunit and the bacterial RNases Bα (barnase), St, and Bt (28). The second largest subunits of all known prokaryotic and eukaryotic multisubunit RNA polymerases contain 12 regions I–XII) highly conserved segments (29). While both eukaryotic and prokaryotic second largest subunits show extensive similarities within the RNase-like domain (a portion of which is located within region V) not all of the RNase-like domain active site region has been conserved in the prokaryotic polymerase subunit.

Barnase is an endonuclease exhibiting weak base specificity; it cleaves GpG and GpA linkages approximately 100 times faster than other phosphodiester bonds (30). Like most ribonucleases, barnase does not require a divalent cation and at least a portion of the active site. Barnase is an endonuclease exhibiting weak base specificity and attacks the 5'-side of the phosphodiester bond, generating products with 3'-phosphates and 5'-OH. Thus, by analogy it is unlikely that the RNase-like domain in RNA polymerase II directly catalyzes the cleavage reaction. It should also be noted that in the small number of cases we have investigated thus far, the rate of cleavage is slowed when the region near the transcript 3'-end is G-rich. Therefore, given the sequence specificity of barnase, one can imagine that the barnase-homologous region within polymerase serves not as the nuclease domain but as a transcript binding site, located several nucleotides upstream of the 3'-end. This model would fit well with the earlier proposal of Surratt et al. (25), who suggested both the existence of a product binding site and the possibility that transcript cleavage occurs by strain-induced hydrolysis. SII (or GreA) could facilitate transcript strain, perhaps by altering the configuration of the RNase-like domain and the domain that positions the transcript 3' terminus within the site of polymerization. Alternatively, movement of the RNase-like domain upstream along the transcript may be required to position the nuclease site. If this were true then based on the hypothesized sequence specificity of the RNase-like domain, polymerase might be reluctant to cleave through G-rich regions, as we have observed. In this interpretation, the nuclease site may include regions within the polymerase that participate in the polymerization reaction.

As mentioned previously, transcript cleavage in prokaryotic ternary complex requires the bacterial GreA protein. While GreA does not contain any obvious sequence similarity to SII, GreA does contain two putative nucleic acid binding domains (31). Moreover, GreA was first characterized as a suppressor of pleiotropic mutations within the rif' region of the second largest polymerase subunit of E. coli (32). The probable involvement of GreA in the regulation of bacterial gene expression during the elongation phase and the similarities between the truncation reaction in prokaryotic and eukaryotic systems further support the notion that elongation factor SII is involved in the transcriptional control of gene expression in eukaryotes. In the accompanying paper (33) we determine the SII-facilitated cleavage products of a different class of ternary complexes, namely those which have become blocked in elongation at intrinsic arrest sites. We also investigate cleavage of ternary complexes that are artificially stalled within regions of DNA that partially mimic an intrinsic arrest site.

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