Template End-to-End Transposition by RNA Polymerase II*

Michael G. Izban‡§, M. Angela Parsons¶, and Richard R. Sinden¶

From the ‡Department of Obstetrics and Gynecology, Sealy Center for Molecular Science, the University of Texas Medical Branch, Galveston, Texas 77555-1062 and the ¶Center for Genome Research, Institute of Biosciences and Technology, Department of Biochemistry and Biophysics, Texas A & M University, Houston, Texas 77030-0030

On 5'-template strand protruding templates, promoter-initiated run-off transcription by RNA polymerase II generates discrete, 15–16-nucleotide (nt) longer than expected products whose production is abrogated by elongation factor SII (Parsons, M. A., Sinden, R. R., and Izban, M. G. (1998) J. Biol. Chem. 273, 26998–27008). We demonstrate that template terminal complexes produce these RNAs and that transcript extension is a general and salt-sensitive (250 mM) feature of run-off transcription. On 5’-overhang templates the extended run-off transcripts appear to be retained within an RNA-DNA-enzyme ternary complex, and SII facilitates resumption of transcript elongation via a dinucleotide truncation intermediate. Moreover, on one of the 5’-overhang templates, the initially extended complexes spontaneously resumed transcript extension and were uniquely resistant to salt (250 mM) challenge. However, SII did not facilitate this long distance extension on all template ends. Run-off transcripts on a blunt-ended template were initially extended by 2–11 nt (roughly in 2-nt increments); SII addition either before or after extension resulted in the accumulation of a 4–5-nt extension product. Based on these findings, we propose that the initial and continuously extended RNAs reflect intermediates and successful completion of template end-to-end transposition (template switching) by RNA polymerase II, respectively. Both the template end sequence and structure influenced the success of such an event.

The control of gene expression is complex, and RNA polymerase II is regulated both at the initiation and elongation phases of mRNA synthesis (see Ref. 1 for recent review). Transcription factors that influence the efficiency of elongation, either by interacting with or by covalently modifying polymerase subunits, have been identified biochemically (2–8). Using in vitro systems, one such RNA polymerase II-binding protein (termed elongation factor SII) has been shown to potentiate the removal of misincorporated nucleotides (9) and enhance readthrough of nucleoprotein structures (10, 11) and template sequences (intrinsic arrest sites) that can cause RNA polymerase to adopt an elongation-incompetent configuration (12–16). Moreover, there is compelling evidence that SII facilitates a catalytic site-dependent transcript hydrolysis reaction intrinsic to RNA polymerase (17, 18). Within arrested complexes, nascent transcript cleavage occurs 7–18 nt upstream of the RNA 3’-terminus and transcription resumes from the newly created 3’-end (19–21). Cleavage by artificially stalled transcriptionally competent complexes (19, 20, 22) and transiently stalled complexes containing 3’-end mismatches (9) occurs principally in a dinucleotide increment.

Over the past few years much effort has focused on defining the structural and biochemical properties of artificially and intrinsically halted eukaryotic (see for instance Refs. 17, 19, and 23–29) and prokaryotic (see for instance Refs. 30–47) RNA polymerases, and new structural models for the ternary complex have emerged (see Refs. 1 and 48 for a recent review). In accordance with the early revisions of Chamberlin (33), new models postulate that the overall stability of the ternary complex is influenced both by binding of the nascent RNA to a product-binding site(s) within the enzyme and by bilateral template interactions between the active center of polymerase and the template DNA from +2 to −18 (relative to the site of polymerization) and a DNA “clamp” and double-stranded template 7–10 bp downstream of the polymerization site. More recent studies indicate that the ternary complex contains an invariant approximate 8-nt RNA/DNA “hybrid” within the catalytic center located just upstream of the site of catalysis. Although the heteroduplex is not a major contributor in overall complex stability, the relative strengths of the downstream DNA contact and the RNA/DNA hybrid thermodynamically influence the lateral stability of the ternary complex (44, 46, 47). According to this model, transcriptional arrest by RNA polymerase II is viewed as a thermodynamic minimum where the coupled instability of the transcript 3’-end heteroduplex and downstream template contact result in an upstream shift of the entire enzyme thereby backtracking the transcription bubble to accommodate a more energetically favorable heteroduplex. This simple model is also consistent with the apparent ability of RNA polymerase II ternary complexes to oscillate between transcriptionally competent and incompetent configurations. Arrested complexes have a limited capacity to spontaneously resume transcription (49), and stalled complexes containing 3, 4, or 5 consecutive 3’-terminal UMPs (a common feature of attested complexes) remove nascent transcript in progressively larger cleavage increments and yet retain complete or partial elongation competency (19, 20).

However, current models, based on the biochemical properties of static complexes, cannot account for all of the experimentally determined biochemical properties of RNA polymerase and probably underestimate the kinetic intermediates of a dynamically transcribing polymerase molecule (see Refs. 1 and 48 for extensive discussion of this point). Indeed, using certain SII and yeast polymerase subunit mutants, it has been demonstrated that SII-facilitated cleavage and subsequent

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‡ To whom correspondence should be addressed: Dept. of Obstetrics and Gynecology, Sealy Center for Molecular Science, The University of Texas Medical Branch, Galveston, TX 77555-1062. Tel.: 409-747-0086; Fax: 409-747-0475; E-mail: Mizban@utmb.edu.

¶ The abbreviations used are: nt, nucleotides; bp, base pair; ML major.

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readthrough activities at the well characterized human histone H3.3 gene Tα1 intrinsic arrest site can be uncoupled (18, 50).

In an effort to establish additional in vitro assays to access elongation factor-dependent and -independent properties of RNA polymerase II, we recently investigated the effects of (CNG) triplet repeat tract sequences on the transcriptional properties of RNA polymerase II. During the course of this work, we uncovered a novel property of run-off transcription on linear templates, namely the production of discrete 15–16-nt longer than expected products whose synthesis was abrogated by elongation factor SII (61). Here we show that production of longer than expected products is a general feature of run-off transcription, and we present evidence that these RNAs represent run-off transcript extension products formed by template terminal complexes. Based on the effects of salts and elongation factor SII on oversized product formation and “stability,” we speculate that at some template ends, terminal RNA polymerase II complexes may retain their run-off transcript and continue transcript elongation after switching to another linear template end. The 15–16-nt extension reaction appears to represent a transitional stage in this process.

EXPERIMENTAL PROCEDURES

Materials—All fast protein liquid chromatography-grade non-radioactive nucleotides were obtained from Amersham Pharmacia Biotech. [α-32P]CTP (800 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) were obtained from NEN Life Science Products, whereas restriction and modifying enzymes were purchased from New England Biolabs or Boehringer Mannheim, respectively.

Assembly and Purification of Transcriptionally Stalled Ternary Complexes—In vitro transcription reactions were performed using previously described templates and procedures (61). Three different HeLa cell nuclear extract preparations were used during the course of this work, and the reactions shown in Fig. 1, Figs. 2B, 2C, 3, 4, 5, and Fig. 2A are grouped accordingly.

Transcript Elongation Reactions—Elongation reactions were performed using Sarkosyl-rinsed ternary complexes stalled within a slightly modified adenoviral major late (ML) promoter after UMP incorporation at position +20 (U20 complex). This procedure (51), which incorporates transient Sarkosyl exposure and subsequent gel filtration, changes the reaction buffer to 20 mM Tris-HCl, pH 7.9, 62.5 mM KCl, 0.2 mM EDTA, and 1 mM dithiothreitol and removes detergent-labile non-specific DNA-binding proteins and presumably any elongation factors that may be retained within the stalled complex. Typically, elongation time course reactions were performed in sufficient initial volumes such that 30–μl aliquots could be withdrawn at each time point. Prior to resuming transcription, the reaction mix was supplied 1 mM NTPs and equilibrated to 37 °C for 3 min before the addition of MgCl2 to 7.8 mM. The 30–μl reactions were stopped at specific time points by addition of 70 μl of Stop Mix (20 mM EDTA, 0.2% SDS, and 0.2 mg/ml Proteinase K). In reactions containing recombinant human elongation factor SII, 4 μg/ml recombinant protein was added either prior to equilibration or at 37 °C or shortly after transcription was resumed. Recombinant SII was purified as described previously (22). To maintain buffer and substrate parity when testing the effect of KCl and SII on run-off transcription product formation (Figs. 3–5), water was added to non-KCl supplemented reactions up to 10% of the final reaction volume. Transcription products were processed and fractionated on sequencing gel containing 37 °C or shortly after transcription was resumed. Recombinant SII was purified as described previously (22). To maintain buffer and substrate parity when testing the effect of KCl and SII on run-off transcription product formation (Figs. 3–5), water was added to non-KCl supplemented reactions up to 10% of the final reaction volume. Transcription products were processed and fractionated on sequencing gel containing 7.8 mM urea as described previously (51). The RNA markers (base hydrolysis ladders) were prepared by resuspending purified and precipitated transcripts derived from 200-μl run-off transcription reactions in 20 μl of 50 mM sodium carbonate, pH 9.1, 2.0 mM EDTA followed by a 15-min incubation at 90 °C. The ladders were ethanol-precipitated after the addition of 80 μl of TE and 50 μl of 7.5 M ammonium acetate. Typically, 5% of the reaction mixture was loaded per gel lane.

Gel Analysis—Gels were analyzed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA) and exposed to Kodak X-AR film overnight with an intensifying screen (Corning, Lightning Plus). The percentage of a particular set of run-off transcripts were calculated as described previously (61).

RESULTS

A synchronized in vitro transcription system was used to monitor the production of run-off transcription products. Promoter-initiated RNA polymerase II ternary complexes stalled after insertion of UMP at template position +20 (U20 complex) are purified, following transient exposure to the detergent Sarkosyl, by gel filtration chromatography (see “Experimental Procedures” and Ref. 52). These “core” complexes, which are apparently devoid of the known elongation factors, resume transcript elongation when supplied NTPs and Mg2+. Using this approach it was previously demonstrated that run-off transcription on mixed DNA sequence templates generates three classes of transcription products (53). The first class were formed by RNA polymerase that halts polymerization 6–12 nt upstream of the template end. These complexes survive purification by gel filtration. Subsequent addition of elongation factor SII facilitates transcript truncation 7–14 nt upstream from the transcript 3′-end. The second class of transcripts is produced by polymerase that transcribes to within 4 nt of the template strand end. These gel-filtered complexes are principally unresponsive to SII. Thus, template terminal complexes apparently terminate transcription. All template ends generated a third class of transcripts at low but detectable levels. These RNAs were 2–4 nt longer than expected relative to the end of the template strand end.

Time Dependence of Longer Than Expected Transcript Production on HindIII-linearized pML(CTG)17, EcoRI-digested pML(CAG)12, and the FspI-linearized Mixed Sequence pML20 Template—In the accompanying paper (61) we demonstrated, as expected, that run-off transcription on the 5′-overhanging pML(CTG)17 and pML(CAG)17 templates generated predominantly “full-length” run-off transcripts (see Fig. 1, A and B, respectively). In addition both templates generated low levels of transcripts roughly 12 and 20 (pML(CTG)17) and 8 (pML(CAG)17) nt shorter than expected. Unexpectedly, ~4% of the run-off transcripts were approximately 15 or 18 nt longer than expected (the exact lengths are determined below). Since no oversized products longer than 2–4 nt were detected in previous work on a variety of template ends, we initially considered the possibility that transcript extension occurred during transcription of the triplet repeat tracts. However, the pML(CTG)17-derived longer than expected products were promoter-initiated transcripts containing the expected length of triplet repeat tract sequence (61).

In the course of investigating whether the oversized products were the result of slippage during transcription of the triplet repeat tract, we found that the percentage of 15–18 nt longer than expected transcripts varied depending on transcription reaction time and reaction salt concentration. These points are illustrated in Figs. 1 and 2. Time course transcription studies demonstrated that the production of oversized transcripts was delayed relative to the time required for the majority of complexes to reach the pML(CTG)17 (Fig. 1A, lanes 1–3) and pML(CAG)17 (Fig. 1B, lanes 1–6) template ends. One-minute reactions (Fig. 1A, lane 2, and Fig. 1B, lanes 3) were essentially devoid of the 15–16-nt longer than expected products even though the majority of templates were elongated to within 20 nt of the template end. Continued incubation through 2 min on pML(CTG)17 (Fig. 1A, lane 3) or 5 min on pML(CAG)17 (Fig. 1B, lanes 4–6) resulted in the accumulation of oversized products. On the pML(CAG)17 template, the accumulation of full-length transcription products was essentially unchanged after 1.5, 2, and 5 min, whereas the percentage of 15–18-nt longer than expected products increased from 5 to 11%. These data indicate that transcript extension occurred after polymerase reached the end of the linear template.

In our previous examination of the biochemical properties of run-off transcription complexes generated on mixed DNA sequence linear templates, short elongation times (1–2 min) and...
subsequent gel filtration were used to purify “run-off” ternary complexes (53). Using the previously characterized FspI-linearized pML20 template, we reexamined the effect of extended elongation times on the production of longer than expected transcripts (Fig. 2A). As expected, after a 1- or 2-min transcription reaction (Fig. 2A, lanes 2 and 3, respectively) the majority of ternary complexes either transcribed to the template end (indicated as run-off) or halted transcription 6–9 nt upstream.

**Fig. 1.** Time-dependent accumulation of the longer than expected transcription products on the pML(CTG)$_{17}$ and pML(CAG)$_{17}$ templates. Transcription reactions were performed on pUC-based HindIII-linearized pML(CTG)$_{17}$ (A) and EcoRI-linearized pML(CAG)$_{17}$ templates (B). A, a single 90-μl reaction mixture containing transcription complex stalled at position +20 was supplied with NTPs to 1 mM and equilibrated to 37 °C for 5 min prior to resumption of elongation by addition of 7.5 mM MgCl$_2$. One-third of the reaction was withdrawn and stopped at the indicated times in 70 μl of 20 mM EDTA, 0.2% SDS. The RNAs were purified and fractionated on a 10% (acrylamide/bisacrylamide (29:1)) sequencing gel. B, transcription time course reactions (180 μl) were performed on the EcoRI-linearized pML(CTG)$_{17}$ template as described in A, except that the reaction salt concentration was reduced from 62 to 52 mM KCl by the addition of water. RNAs were purified and fractionated on a 7% (acrylamide/bisacrylamide (19:1)) sequencing gel run until the xylene cyanol had migrated 37 cm.

**Fig. 2.** Longer than expected transcript formation on the FspI-linearized pML20 template and high resolution mapping analyses. A, transcription time course reaction on the linearized pML20 template (lanes 2–6) was performed as described in Fig. 1B. The products displayed in lane 1 were generated during a 5-min chase reaction that was supplemented with 0.3% Sarkosyl 15 s after Mg$^{2+}$ addition. Transcripts were resolved on a 5% (acylamide/bisacrylamide (19:1)) sequencing gel (25 x 37 cm) run until the xylene cyanol had migrated 37 cm. B–C, base hydrolysis ladders were generated from 5-min run-off transcription reactions using linearized pML20 (B, lanes 2 and 4), pML(CAG)$_{17}$ (B, lane 9), and pML(CTG)$_{17}$ (C, lane 1) templates as described under “Experimental Procedures.” Transcripts from a 5-min chase reaction in the absence (C, lane 2) or presence of 120 ng of rSII (rSII was added 1 min after the transcription start time lanes 3 and 10 (B) and lane 3 (C)) were fractionated adjacent to their respective base hydrolysis ladder. DNA sequencing ladders are shown in lanes 1 and 5–8 (B). The purified reaction products were resolved on 5% (acylamide/bisacrylamide (19:1)) sequencing gels (45 x 70 cm). The gels in B and C were run until the xylene cyanol had reached 70 or 60 cm, respectively. The expected full-length run-off transcripts are 213 (pML20), 210 (pML(CAG)$_{17}$), and 185 nt (pML(CTG)$_{17}$). The salient base hydrolysis and run-off transcription products are indicated in between lanes 2 and 3 of B and in the right and left margins of B and C, respectively.
Also as reported, 0.3% Sarkosyl selectively inhibited continued transcription by complexes that would normally transcribe to the template end (Fig. 2A, lane 1). However, extending the reaction time resulted in a concurrent reduction in transcripts at or near the template end and an accumulation of predominantly 5- and 7-nt longer than expected products (Fig. 2A, lanes 4–6). After 15 min (Fig. 2A, lane 6) approximately 40% of the total run-off transcripts were 5 and 7 nt longer than expected. Similar results were observed on another previously characterized 3’-recessed template strand end template (BglI-digested pML20) which produced primarily a 16-nt longer than expected product (data not shown). Thus, longer than expected transcript formation appears to be a general feature of run-off transcription.

High Resolution Mapping of Run-off Transcription Products—All transcript length assignments were determined by high resolution mapping analyses. Typically, run-off transcripts were subjected to partial base hydrolysis and fractionated alongside a portion of the non-hydrolyzed transcript preparation. The hydrolysis ladders contain easily identifiable patterns since RNA NpG phosphodiester bonds are slightly more labile (54). For example, the relative intensities and lengths of hydrolyzed run-off transcripts derived from the FspI-linearized pML20 template (Fig. 2B, lane 2) were consistent with the transcript 3’-end sequence (5’-C170GAAGAGGCCGGCCAGGGAUC224GCCUUCCAAACGUGUC239) where underlined 3’-terminal RNAs are indicated in Fig. 2. Also note that the distribution of run-off transcripts remains easily discernible after limited base hydrolysis (compare Fig. 2A, lanes 4 and 5, with Fig. 2B, lanes 2 and 4). On the FspI-linearized pML20 template, the exact distribution of oversized transcripts varied slightly from reaction to reaction (compare Fig. 2B, lanes 2 and 4) and between nuclear extract preparations (compare Fig. 2A, lanes 4 and 5, with Fig. 5, lane 6, which produced predominantly 2-, 7-, and 9-nt overextension products). Although the reason behind this heterogeneity remains unknown, the extension products were always a subset of the transcripts shown in Fig. 2B (lane 2). These run-off transcripts were extended by 1, 2, 5, 7, 9, or 11 nt and represented approximately 2, 4, 25, 8, 1.6, and 0.6%, respectively, of the total (+204 to +224 nt) run-off RNAs. We assume that the heterogeneity of oversized transcripts represents intermediates along a common extension pathway.

As expected for 5’-overhang template ends, high resolution mapping of the pML(CTG)17 (Fig. 2C, lane 2) and pML(CAG)17 (Fig. 2B; lane 17) transcription products demonstrated that the majority of the run-off transcripts were either 4 nt shorter than expected (+181) or full-length (+210), respectively. A minor fraction of complexes halted transcription 10 and 11 nt (pML(CAG)17, Fig. 2B, lane 9, and additional data not shown) or 16, 17, and 23 nt (pML(CTG)17, Fig. 2C, lane 2) upstream of the template strand end. The longer than expected transcripts were extended 12 (pML(CTG)17) or 16 nt (pML(CAG)17) past the template strand end. No variation in the extension lengths was observed using several different nuclear extract and template preparations.

The data in Figs. 1 and 2 also serve to illustrate that the extension reaction was sensitive to reaction salt concentrations. For example, approximately 11% of the pML(CAG)17 run-off transcripts were extended by 16 nt after a 5-min reaction performed at 25 mM KCl (data not shown) or 52 mM KCl (Fig. 1B, far right lane). At our standard reaction salt concentration (59 mM), this percentage was reduced to approximately 3% (Fig. 2B, lane 9). At 100 mM KCl the product was barely detected (data not shown) and completely absent at 250 mM KCl (see below; Fig. 4, lane 5). Transcript extension on the pML(CTG)17 and pML20 templates shared similar salt sensitivity.

As previously reported (53), we also observed a minor fraction of run-off transcription products that were 1–4 nt longer than expected. These RNAs are clearly resolved on the pML(CTG)17 (Fig. 2C, lanes 1 and 2) and pML(CAG)17 (Fig. 2B, lane 9) templates. Based on the observation that binary complexes of RNA polymerase II and RNA are capable of adding 1–4 nucleotides to the 3’-end of the RNA (29), we had previously speculated that this class of product was formed via a non-templated nucleotide addition pathway. However, our preparations of U20 complex routinely contain a small percentage of complexes bearing 21–24-nt transcripts (data not shown, see the accompanying paper (61) and Ref. 22). Thus, it is possible that the 1–4-nt extension products represent run-off transcription by these “aberrantly” initiated polymerase complexes. The following observations are consistent with this interpretation. First, the percentage of “contaminants” within the U20 complex preparations and the 1–4-nt longer than expected run-off transcription products were similar (data not shown). Second, the accumulation of the 1–4-nt longer than expected run-off products mimicked the kinetics of full-length run-off transcript production (see Figs. 1B, and 2A). Third, both the full-length and 1–4-nt longer than expected transcripts were present when elongation was performed at 250 mM KCl (high resolution data not shown, but see Fig. 5, lane 1) or when elongation factor SII was included in the reaction mix (Fig. 2B, compare lanes 9 and 10 and Fig. 2C compare lanes 2 and 3).

These data also show that elongation factor SII and elevated reaction salts altered the final distribution of full-length run-off transcripts. Relative to the major run-off transcript, 1–4-nt shorter transcripts were produced. The exact distribution varied between salt and SII supplementation (see for instance Fig. 5, lanes 1–7, and additional high resolution data not shown). At all template ends, elevated salt concentrations produced run-off transcripts that were distributed, essentially evenly, between the major run-off product and 1- and 2-nt shorter RNAs (high resolution data not shown but see Fig. 5, lane 1). We assume that elevated reaction salt concentrations destabilizes the ternary complex as transcription approaches to within 2–3 nt of the template end. A similar transition in ternary complex stability was detected during transcription in the presence of the detergent Sarkosyl (Fig. 2A, lane 1 and Ref. 53). The effect that SII had on the exact distribution of run-off transcripts was more variable. There is no reason a priori to suppose that elongation factor SII would destabilize the ternary complex as it approaches the template end. Rather, we interpret these data to indicate that template terminal complexes remain bound to the template end and remain SII-responsive. In this regard, it has previously been shown that when U20 complex was supplied SII and a subset of NTP that only allows extension of cleaved transcripts back to the original stall site 20-, 19-, and 18-nt transcripts are generated (22). Although essentially all of the “cycling” U20 complexes remained transcriptional engaged, the percentage and/or half-life of SII-responsive template terminal complexes is unknown.

The Effect of Salt Concentration and Elongation Factor SII on Run-off Transcript Extension—Because different nuclear extract preparations and different reaction salt concentrations caused slight variability in extension product formation, the following transcription reactions were performed using the same nuclear extract preparation and, unless otherwise indicated, contained 52 mM KCl. As indicated above, addition of 250 mM KCl prior to resumption of transcription completely inhibited the accumulation of the 12- and 16-nt longer than expected products on the pML(CTG)17 (Fig. 3, lane 5) and pML(CAG)17
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FIG. 3. Effects of salt and elongation factor SII on the transcript extension reaction. The transcription reactions on the HindIII-linearized pML(CTG)$_{17}$ template were derived from one large pool of U20 complex. Individual 30-µl reactions were supplied 1 mM NTPs (lanes 1–5) and stopped 1 lane 1 or 5 lanes 2–5 min after Mg$^{2+}$ addition. The reactions mixtures corresponding to lanes 2–5 were additionally supplemented with 120 ng of rSII or KCl to 250 mM either prior to (lanes 4 and 5, respectively) or 1 min after (lanes 2 and 3, respectively) Mg$^{2+}$ addition. The reaction conditions for lanes 6 and 7 were identical to lanes 1 and 3, respectively, except that a single 60-µl reaction was chased for 2.5 min and half, the reaction was simultaneously stopped or supplied with KCl. Similarly, a 120-µl reaction was chased for 2.5 min, and 30 µl of the reaction mixture was stopped (lane 8) while the remaining 90 µl received 360 ng of rSII and subsequently stopped after 15 (lane 9), 30 (lane 10), or 60 (lane 11) s. One-half of the purified transcripts was resolved as described in Fig. 1A. Run-off transcripts are indicated at left. (Fig. 4, lane 5) templates, respectively. If run-off transcripts were extended by template terminal complexes, then KCl addition should have similar effects after the majority of the polymerase molecules had reached the template end. Indeed, extension product formation was inhibited when KCl (Figs. 3 and 4, lane 3) was added after an initial 1-min reaction (shown in Figs. 3 and 4, lane 1), where the majority of ternary complexes had reached the template end but before significant synthesis of oversized products. Note also that essentially all of the active stalled U20 complexes quantitatively chased “to the template end” in the KCl-supplemented reactions (data not shown). These data are consistent with the notion that elevated salt inhibited the ability of template terminal complexes to extend their run-off transcripts.

SII, on the other hand, might facilitate removal of the extended portion of the transcript via its known transcript truncation stimulatory activity (21, 22). As expected, the 12- and 16-nt extension products were absent when SII was supplied either prior to (Figs. 3 and 4, lanes 4) or 1 min after (Figs. 3 and 4, lanes 2) transcription was resumed. However, the percentage of full-length run-off RNAs in the reactions supplied SII (Figs. 3 and 4, lanes 2 and 4) were reduced relative to that observed in the presence of 250 mM KCl (Figs. 3 and 4, lanes 3 and 5). Compared with the percentage of pML(CTG)$_{17}$-derived transcripts formed in the presence of KCl (Fig. 3, lanes 3 and 5), only 35% of the run-offs were present in the SII-supplemented reactions (Fig. 3, lanes 2 and 4). Moreover, this reduction was accompanied by a concomitant increase in longer transcripts whose rate of extension approximated the template-dependent polymerization rate of these preparations of RNA polymerase II. For example, in 5-min reactions (Fig. 3, lanes 3 and 4) the longer RNAs were 1000 nt in length, and their distribution was similar to the minor fraction of similarly sized transcription products (Fig. 3, lane 5) generated either from nonspecific RNA polymerase II initiation events (53) or from promoter initiation events on uncut templates. Similarly, on the pML(CAG)$_{17}$ template, SII supplementation (Fig. 4, lanes 2 and 4) reduced the amount of run-off transcripts to 85% that observed with KCl supplementation (Fig. 4, lane 5). The portion of the gel containing the longer RNAs is not shown.

To explore further the mechanism by which SII stimulated run-off transcript extension, we tested the effects of SII (and 250 mM KCl) on preexisting oversized transcripts. For this, an initial 2.5-min transcription reaction was performed on the pML(CTG)$_{17}$ (Fig. 3, lane 6 and 8) and pML(CAG)$_{17}$ (Fig. 4, lane 6) templates prior to SII or KCl addition. Inspection of the distribution of run-off transcripts on the latter templates, 15 (Fig. 4, lane 8), 30 (Fig. 4, lane 9), and 60 s (Fig. 4, lane 10) after SII supplementation indicated that the preexisting oversized transcripts were truncated in the presence of SII. Based on their distribution over time, it appears that they were shortened initially by 2 nt prior to their progressive disappearance. In contrast, the amount of full-length run-off transcripts was essentially unaffected by SII addition (Fig. 4, lane 8–10). Similar results were obtained when SII was added to 2.5-min pML(CTG)$_{17}$ reactions (Fig. 3, lanes 9–11). High resolution mapping indicated that these oversized transcripts were rapidly shortened by 4 nt prior to their progressive disappearance (data not show). We could not discern whether transcript truncation occurred via multiple 2-nt cleavage increments or by a single 4-nt cleavage event (additional data not shown). That the over-sized products were truncated in a short cleavage
increment suggests that their transcript termini were positioned within the catalytic site of an RNA polymerase II ternary complex (19) or a binary complex of RNA and RNA polymerase II (29). However, transcript extension of RNA within a binary complex is limited to 2–4 nt. Taken together, these data strongly suggest that the 12- or 16-nt extension products were retained in ternary complex and that SII facilitated the resumption of transcription via a transcript truncation pathway.

The effect of elevating the reaction salt concentration on complexes bearing the extension products was unanticipated. As expected, the levels of full-length and 16-nt extension products generated on the pML(CAG) template after a 2.5-min transcription reaction (Fig. 4, lane 6) were essentially unaltered following KCl addition (Fig. 4, lane 7). The portion of transcripts that had been extended (16%) was also in agreement with the 15% reduction in full-length transcripts observed in SII-supplemented reactions (Fig. 4, lanes 2 and 4). In contrast, the pML(CTG)17-derived 12-nt extension products did not persist after salt challenge (Fig. 3, compare lanes 6 and 7). This was not the result of an RNase contaminant within the reaction mixture since the level of full-length run-off was unaltered following salt treatment. Furthermore, the amount of 12-nt extension product in the 2.5-min reaction cannot account for the 35% reduction in full-length run-off RNAs in the SII-supplemented reactions. Thus, continued extension of the 12-nt longer than expected RNA was not dependent on exogenously added elongation factor SII. These data indicate that the 12-nt extension products were retained within a salt-resistant “paused” RNA polymerase II ternary complex.

The effect of elevated KCl concentrations on the production of the FspI-linearized pML20-derived 2-, 7-, and 9-nt extension products was identical to that observed with the pML(CAG)17 template. The production of oversized RNAs was salt-sensitive (Fig. 5, compare lanes 1, 3, 4, and 6, and additional data not shown), and salt had no effect on preexisting oversized run-offs (Fig. 5, compare lanes 6 and 7). In contrast to the effects of SII on the pML(CTG) and pML(CAG) templates, longer than expected transcripts were produced on the FspI-linearized pML20 template in the presence of SII (Fig. 5, lane 2) or when SII was added 1 min after the resumption of transcription (Fig. 5, lane 5). However, the oversized transcripts were only extended by 4 or 5 nt. Similar results were observed in run-off reactions that produced primarily 5- and 7-nt extension products (see Fig. 2B, compare lanes 3 and 4). The 7- and 9-nt extension products generated in the 4-min reaction (Fig. 5, lane 6) appeared to be converted upon SII addition to the transcripts extended by 4 and 5 nt (Fig. 5, lanes 8–10) suggesting that the 7–9-nt extension products were intermediates in the formation of the shorter extension products. Also in contrast to the 5’-overhanging templates, the amount of total run-off transcripts was essentially identical irrespective of the salt (or SII) supplementation regiment (Fig. 5, lanes 1–5). These data indicate that this template end cannot support efficient transcript extension beyond the 7–9-nt stage.

One additional point is worth mentioning. As indicated above, RNA polymerase II has a strong tendency to arrest transcription 6–9 nt upstream of the blunt-ended FspI-linearized template end (Fig. 5, lane 4). As expected, addition of SII (Fig. 5, lanes 2, 5, and 8–10) facilitated readthrough by these complexes to the template end apparently via a transcript truncation pathway since the distribution of arrested complexes (Fig. 5, lane 6) was shifted upstream after SII addition (Fig. 5, lanes 8–10). The efficiency of transcription through this region was also increased at elevated KCl concentrations (Fig. 5, lanes 1, 3, and 7). Similarly, SII (Fig. 2B, lane 10, and Fig. 2C, lane 3) and elevated KCl (data not shown) enhanced readthrough of the minor fraction of complexes that arrest prior to the pML(CTG)17 and pML(CAG)17 template ends, respectively.

**DISCUSSION**

In the accompanying study (61) we uncovered a novel feature of run-off transcription, namely the production of discrete roughly 16-nt longer than expected transcripts. By examining the properties of RNA polymerase II at a variety of linear template ends as a function of time and in response to elongation factor SII and reaction salt concentrations, we showed that transcript extension is a general feature of template terminal complexes. Our results strongly indicate that the initially extended transcripts are retained within ternary complex and that at some template ends these complexes can resume ectopic transcript extension.

**Run-off Transcription**—Previous studies have demonstrated that as RNA polymerase II approaches the end of linear templates, it may arrest 6–10 bases upstream of the template end or continue to transcribe to within 4 bases of the template strand end and terminate transcription (53). The arrested complexes remain associated with the template during gel filtration purification. Subsequent addition of elongation factor SII facilitates transcript truncation 7–14 nt upstream from the template 3’-end. As expected, we show that elongation factor SII facilitates the arrested complexes to readthrough to the template end via a truncated transcript intermediate (Figs. 5, lanes 8–10). In addition, in the absence of exogenously added SII, “arrested” complexes spontaneously resumed transcription upon prolonged incubation with NTPs (see in particular Fig. 2A, lanes 2–6). Similar observations have been made with complexes arrested at canonical arrest sites within circular templates (see for instance Ref. 49) and support the notion that arrested complexes are in equilibrium between transcriptionally active and inactive states (19, 49). The efficiency of factor-independent readthrough to the template end was also increased at elevated salt concentrations (Fig. 5, lane 7, and additional data not shown). Based on previous work (53), we do not believe that elevated salt destabilized template end nucleoprotein structures, which might block transcription to the template end and provoke the arrested configuration (11). Rather, the kinetic barrier to elongation that can occur 6–9 nt upstream of some template ends (Fig. 2) might represent a conformational transition to a transcriptionally competent complex that lacks downstream template contacts (discussed...
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Elevated salt concentrations could influence this transition by affecting protein/protein, nucleoprotein, and/or nucleic acid interactions. For example, increasing the reaction salts may enhance the rate of formation or stability of a properly positioned RNA/DNA hybrid within the catalytic center. This interpretation would be consistent with the notion that the strength of the RNA/DNA hybrid at and just upstream of the catalytic site influences the ability of RNA polymerase to adopt an arrested configuration (19, 44). Our data also suggest that polymerases that have transcribed to within 4 bases of the template strand end remain template-engaged and SII-responsive (Fig. 2B, lane 10, Fig. 2C, lane 3, and additional data not shown). The percentage and relative stability of the engaged template terminal complexes remain to be determined.

These results apparently contradict previous studies showing that template terminal complexes were unresponsive to SII and that a 72-nt run-off transcript could be separated from the DNA template during gel filtration chromatography (53). A more plausible explanation would be to assume that the run-off ternary complexes dissociated from the template end during the 5-min chromatographic step. Consistent with this interpretation, we find that template terminal complexes generated on linear templates immobilized to agarose beads quantitatively retain full-length run-off products following centrifugation of a 1.5-min elongation reaction (data not shown).

**Run-off Transcript Extension**—Clearly, our most striking observation was that template terminal complexes produce longer than expected products that, depending on the template end, were initially extended by 2–16 nt but nevertheless retained within ternary complex. Depending on the template end sequence/structure, SII facilitated either continued extension of these transcripts (Figs. 3 and 4) or the accumulation of a shorter extension product (Fig. 5). Moreover, under reduced reaction salt conditions, one particular template supported easily detectable partitioning between run-off transcripts and transcripts that were extended past the template end at template-directed RNA polymerase II transcription rate, presumably via a 12-nt extension intermediate (Fig. 3). Although the data set are small and formal proof awaits further analysis, the biochemical features of template terminal RNA polymerase II complexes are consistent with a recent report demonstrating that *Escherichia coli* RNA polymerase template terminal complexes can switch to a new template end and continue nascent transcript extension (55). Of particular relevance to the discussion of our results is that this study defined two separate polymerase/DNA interactions that contributed to the stability of the elongation complex. The catalytic center, which minimally encompasses, relative to the site of polymerization, from −6 to +1 of the template strand and a DNA clamp which interacts with 7–12 bp of adjacent downstream duplex DNA. The latter interaction was necessary for maintenance of a salt-resistant elongation complex and required for long distance processive elongation (55). That RNA polymerase II might carry out a similar reaction was not entirely unexpected since it is well established that purified RNA polymerase II will efficiently initiate from linear template ends providing that these templates contain an “on ramp” consisting of a template strand polymeric dC overhang (dc-tail) (56, 57).

On all the template ends we tested, the accumulation of the 2–16-nt oversized products was time-dependent (see in particular Fig. 2A) and salt-sensitive (Figs. 3 and 4, lane 5, and Fig. 5, lane 1). In the context of a template-switching model, we speculate that the time dependence reflects the kinetics of secondary template end acquisition by template terminal complexes. Elevating the reaction salt concentration to 250 mM KCl abrogated the production of the 2–16-nt extension products presumably either by affecting the stability of template terminal complexes or by altering the efficiency of secondary template acquisition or both. Consistent with the former possibility, at elevated salt concentrations transcription becomes less efficient as polymerase approaches to within 4–5 bases of the template strand end (Fig. 5, compare lanes 6 and 1, and additional data not shown). Our results also strongly suggest that the particular set of extension products must reflect certain structural and/or sequence variations in the template termini. Consistent with this notion, in contrast to the 12-nt extension product produced on the *Hind*III-linearized pML(CTG)$_{17}$ template, 7- and 18-nt longer than expected transcripts were produced on templates linearized at an adjacent upstream *Pst*I site (data not shown). Moreover, the effects of SII and KCl on the production (and stability) of the *Pst*I-derived oversized products were similar to that observed with the *Eco*RI-linearized pML/CAG)$_{17}$ template (data not shown).

The accumulation of 2–16-nt extension products also indicates that RNA polymerase II encounters a barrier to elongation early after “reinitiating” on the secondary template. On the *Eco*RI-linearized pML/CAG)$_{17}$ template, this barrier, which occurred after an initial 16-nt extension reaction, was essentially insurmountable in the absence of elongation factor SII. Similarly, SII facilitated resumption of transcription by the 12-nt extension complexes generated on the *Hind*III-linearized pML-(CTG)$_{17}$ template. However, continued transcription by these complexes was not dependent on exogenously added SII. Moreover, these complexes resumed transcription when challenged with 250 mM KCl (Fig. 3, lane 7). Thus, it is reasonable to suppose that the salt stability reflects the tendency of these complexes to reacquire appropriate secondary template downstream “clamp” contacts. This rapid conversion to the stable processive configuration would be consistent with the observed tendency of this template to support factor-independent production of longer transcript extension products. Our data also demonstrate that SII enables polymerase to read through these barriers by stimulating transcript truncation within 2–4 nt of the transcript 3′-end (Figs. 3 and 4, lanes 8–10). Guo and Price (58) have reported similar properties for SII on dC-tailed templates that provoke a strong block to elongation early after initiation.

However, not all template ends supported long distance transcript extension. The FspI-linearized pML20 template appeared to be refractory to productive template switching. Whereas this template end supported nucleotide extension by 2, 5, 7, 9, and 11 nt, addition of SII did not facilitate readthrough. Rather, in the presence of SII, complexes bearing 5, 7, 9, or 11-nt extension products appear to truncate their transcripts and become “trapped” intermediates at a 4–5-nt extension stage. Whether this failure is a general feature of extension from blunt-ended templates or due to this particular template end sequence remains to be determined.

The kinetic barriers encountered as polymerase approaches a linear template end and presumably as it acquires a salt-resistant elongation-competent configuration on the secondary template might reflect certain structural intermediates that must occur during the promoter-directed transition from an unstable initiation to a stable elongation complex. Based on the ability of SII to facilitate transcription through these kinetic barriers, it is tempting to speculate that SII may also enhance (or inhibit) the efficiency of promoter clearance *in vivo*. Similar speculative assertions have been made based on conserved putative structural motifs within the SII family members (59) and the biochemical properties of yeast RNA polymerase II ΔRPB9 mutants at the histone H3.3 T1a intrinsic arrest site (18). With respect to this point, it has been demonstrated that
the bacterial GreA and GreB proteins, which are functional homologs of SII, stimulate productive initiation from a certain promoter variant both in vitro (via a short transcript truncation intermediate) and in vivo (60).

Developing a mechanistic appreciation of transcript elongation by RNA polymerase II is essential to our understanding the transcriptional regulation of gene expression. The inherent transcriptional properties of polymerase at the end of linear templates (and at the CTG repeat tract junction within the myotonic dystrophy protein kinase gene and within the defined (CNG) template sequences (see the accompanying paper (61)) should provide useful tools to delineate further the contribution of nucleoprotein interactions on the control of transcript elongation and may assist in further defining the mechanism(s) by which regulatory elongation factors influence this process.

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