The RNA polymerase II ternary complex cleaves the nascent transcript in a 3' → 5' direction in the presence of elongation factor SII

Michael G. Izban and Donal S. Luse¹

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524 USA

The process by which RNA polymerase II elongates RNA chains remains poorly understood. Elongation factor SII is known to be required to maximize readthrough at intrinsic termination sites *in vitro*. We found that SII has the additional and unanticipated property of facilitating transcript cleavage by the ternary complex. We first noticed that the addition of SII caused a shortening of transcripts generated by RNA polymerase II at intrinsic termination sites during transcription reactions in which a single NTP was limiting. Truncation of the nascent transcript was subsequently observed using a series of ternary complexes artificially paused after the synthesis of 15-, 18-, 20-, 21-, and 35-nucleotide transcripts. Transcripts as short as 9 or 10 nucleotides were generated in 5-min reactions. All of these shortened RNAs remained in active ternary complexes because they could be chased quantitatively. Continuation of the truncation reaction produced RNAs as short as 4 nucleotides; however, once cleavage had proceeded to within 8 or 9 bases of the 5' end, the resulting transcription complexes could not elongate the RNAs with NTP addition. Transcript cleavage requires a divalent cation, appears to proceed primarily in 2-nucleotide increments, and is inhibited by α -amanitin. The catalytic site of RNA polymerase II is repositioned after transcript cleavage such that polymerization resumes at the proper location on the template strand. The extent and kinetics of the transcript truncation reaction are affected by both the position at which RNA polymerase is halted and the sequence of the transcript.

[Key Words: RNA polymerase II; elongation factor SII; transcript cleavage]

Received January 13, 1992; revised version accepted April 16, 1992.

Regulation of eukaryotic gene expression at the level of transcript elongation has been well documented (for review, see Spencer and Groudine 1990). The molecular mechanisms involved in the control of elongation, however, are not yet known. *In vitro* studies using RNA polymerase II initiated at natural promoters or at the ends of template molecules by the use of dC tails have demonstrated that there are at least two distinct classes of elongation stimulatory factors. TFIIF and related proteins have been shown to increase the rate of transcript elongation by purified RNA polymerase II, whereas the SII group of factors is required to achieve efficient elongation through intrinsic termination sites (Rappaport et al. 1987; Reinberg and Roeder 1987; Price et al. 1989; Reines et al. 1989; Sluder et al. 1989; SivaRaman et al. 1990; Bengal et al. 1991). Recently, Agarwal et al. (1991) have demonstrated using recombinant human SII that two separate domains within the factor are both required to stimulate elongation. Another factor, termed TFIIX

(Reinberg et al. 1987), which has only been partially purified, contains activities that mimic both TFIIF and SII (Bengal et al. 1991; Izban and Luse, 1992).

A number of DNA sequences that serve as blocks to elongation *in vivo* have been characterized using *in vitro* transcription systems (e.g., see Maderious and Chen Kiang 1984; Kerppola and Kane 1990). These sequences have been termed intrinsic termination sites, although a significant portion of "core" RNA polymerase II ternary complexes (polymerases devoid of the elongation factors mentioned above) can elongate through these regions *in vitro*. Most of the polymerases that become trapped at these sites retain transcript in ternary complex (Reines et al. 1989; Kerppola and Kane 1990; Bengal et al. 1991). A number of different nonphysiological conditions are capable of reducing (e.g., 100 mM NH_4^+ ; see Izban and Luse 1991) or increasing (e.g., suboptimal NTP concentrations; see Kerppola and Kane 1990; Wiest and Hawley 1990) the fraction of polymerases that become blocked in elongation at intrinsic termination sites. Under near physiological conditions, SII stimulates readthrough by core RNA polymerase II at such sites when introduced

¹Corresponding author.

before (Rappaport et al. 1987; Reines et al. 1989; Sluder et al. 1989; SivaRaman et al. 1990) or after (Bengal et al. 1991) the block to elongation. Recently, it has been demonstrated that elongation is blocked initially at intrinsic termination sites even in the presence of SII and saturating NTP concentrations (Izban and Luse 1992). Thus, SII appears to function by releasing halted RNA polymerase II complexes.

Multiple SII-related cDNAs, some of which are expressed in a tissue-specific manner, have been isolated recently from mouse liver libraries (Kanai et al. 1991). Furthermore, an SII homolog in yeast (the PPR2 protein) was originally identified genetically as a transcriptional regulator of another gene (see Hubert et al. 1983 and references therein). These observations, coupled with the known effects of SII on elongation *in vitro*, make it reasonable to suppose that SII-related proteins as a class are involved in gene regulation *in vivo* at the level of transcript elongation. In the course of exploring the effects of SII on paused RNA polymerase II ternary complexes we found that this factor greatly facilitates the 3' → 5' hydrolysis of nascent transcript within ternary complex. Transcript cleavage does not disrupt the ternary complex and is accompanied by the repositioning of the catalytic site such that transcript elongation is resumed at the proper place on the template strand. This cleavage reaction may be part of the normal process of restarting complexes that become blocked during elongation.

Results

We have used the wild-type adenovirus 2 major late (Ad2 ML) promoter and variants thereof as templates for *in vitro* RNA synthesis. Transcription in the absence of one or more NTPs has allowed us to produce a variety of RNA polymerase II ternary complexes artificially paused at discrete positions after the synthesis of between 12- and 35-nucleotide transcripts (Izban and Luse 1991; Linn and Luse 1991). These complexes can be highly purified using a procedure we termed Sarkosyl rinsing, which involves a transient exposure of the paused ternary complexes to Sarkosyl followed by gel filtration in column buffer devoid of Mg^{2+} (Izban and Luse 1991). The chromatographic step separates ternary complexes from the Sarkosyl, nonspecific DNA-binding proteins removed by the Sarkosyl and the NTPs used to initiate transcription. The large majority of paused, Sarkosyl-rinsed RNA polymerases resume synchronous transcript elongation when 7 mM Mg^{2+} and excess NTPs are added (Izban and Luse 1991; see also below). We refer to purified ternary complexes as core RNA polymerase II because Sarkosyl rinsing also appears to remove elongation factors TFIIF, TFIIX, and SII (Hawley and Roeder 1985; Reines et al. 1989; Wiest and Hawley 1990; Izban and Luse 1992; this point is discussed further, below). Because the chase of the ternary complexes is performed with nonlabeled nucleotides, the amount of label incorporated into any given transcript directly reflects the capacity of the RNA

polymerase II to transcribe regardless of transcript length.

Effects of elongation factor SII on the ability of RNA polymerase II to transcribe through and release from intrinsic termination sites

To study the effect of SII at the two well-documented sites within the first intron of the Ad2 ML transcription unit (Maderious and Chen Kiang 1984), we produced Sarkosyl-rinsed early elongation complexes paused at position +12 (see Izban and Luse 1991) downstream of the Ad2 ML promoter on the pSmaF-1 plasmid. We then restarted elongation using either 1 mM NTPs (Fig. 1A, lanes 1–3) or 1 mM ATP, CTP, and GTP and 20 μ M UTP (lanes 4–6), with or without the addition of elongation factor SII. Elongation reactions were performed for either 2 (lanes 1–3) or 10 (lanes 4–6) min. As expected, in the absence of SII many of the RNA polymerases were blocked at the intrinsic termination sites at +120 and +185 of the Ad2 ML gene (lane 1; a longer exposure of the portion of the gel containing the 120-nucleotide transcript is shown at the bottom of Fig. 1A). As reported previously (Bengal et al. 1991), core RNA polymerase II supplemented with SII (lane 2) before chase with 1 mM NTPs reads through these sites much more efficiently (cf. lanes 2 and 1). The same amount of SII used in lane 2 (arbitrarily defined as 1 unit, see Materials and methods) was also used for the other SII supplementation tests reported in this paper unless otherwise indicated. Also as expected (Bengal et al. 1991), RNA polymerases blocked in transcription can resume transcript elongation with high efficiency when SII is added (cf. lane 3 with lanes 1 and 2).

A more dramatic block to elongation has been demonstrated at the Ad2 ML +185 site when transcription is performed at suboptimal GTP concentrations (Wiest and Hawley 1990). We observed a similar effect with suboptimal (20 μ M) levels of UTP (cf. lanes 1 and 6). Although the site of elongation blockage was unchanged at +185, we observed a difference in the location of the block within the +120 region (cf. lanes 1 and 6). The reason for this difference is unknown. When we supplemented the UTP-limiting elongation reactions with SII, we were surprised to find that about the same number of transcripts were generated by elongation blockage at the +185 intrinsic termination site (lane 4) as we had seen in the absence of the factor. In addition, the SII-supplemented elongation reactions produced some transcripts that were shorter than those generated in the nonsupplemented reaction. These RNAs were also produced when SII was added to complexes already blocked (lane 5). Blockage was reduced at the +120 site whether SII was added with the chase NTPs or after the block to elongation had occurred. In this case as well, we observed a significant amount of shorter transcripts in those reactions that had received SII (lanes 4 and 5). Although the shorter transcripts at the +185 site could conceivably have been generated by chase of the complexes previously paused before +185, this explanation seemed less

Figure 1. The effect of elongation factor SII on transcription through and release from intrinsic termination sites. (A) Sarkosyl-rinsed ternary complexes paused at +12 were generated with pSmaF-1 template, and elongation reactions were performed for 2 min after supplying Mg^{2+} and 1 mM NTPs (lanes 1–3) or 10 min after adding Mg^{2+} and 1 mM ATP, CTP, and GTP and 20 μ M UTP (lanes 4–6). SII was added as indicated, either before resumption of transcription (prechase) or after transcription had proceeded for the times indicated above (postpause). In the latter cases, transcription was allowed to continue for an additional 1 (lane 3) or 5 (lane 5) min after SII addition. Transcription reactions performed without SII for 2 (lane 1) and 10 (lane 6) min are also shown. The longest RNAs generated in these reactions were ~600 nucleotides (lanes 1,2) or 300 nucleotides (lane 4,6) in length (data not shown). The purified transcripts were resolved on 6% (19 : 1 acrylamide/bisacrylamide) sequencing gels. Lengths of selected transcripts are indicated at left. A longer exposure of the lower portion of the gel is shown at bottom. (B) Sarkosyl-rinsed

elongation complexes paused predominantly at +15 were generated with pML5-4NR templates, and elongation was performed by supplying the reactions with Mg^{2+} and 1 mM ATP, CTP, and GTP and 20 μ M UTP for the times indicated. SII was added before NTP addition as indicated. SII or UTP to 1 mM was added after an initial chase, as indicated, and elongation was continued for 3 (lane 2) or 5 (lane 7) min. The purified transcripts were resolved on a 10% (29 : 1 acrylamide/bisacrylamide) sequencing gel. The lengths of the relevant transcripts are indicated at left.

likely for the shortened transcripts at +120, as there were essentially no complexes stalled at positions upstream of +120 before the addition of SII (additional data not shown).

We also examined the effects of SII at a second intrinsic termination site. The pML5-4NR plasmid (Izban and Luse 1991) contains a tetrameric repeat of a 185-bp DNA fragment cloned downstream of the Ad2 ML promoter. Each repeat contains a region similar in sequence to the minimal termination site within the *c-myc* terminator (Kerppola and Kane 1990). We generated artificially paused early elongation complexes (C15/U18 complexes; see below) that were then purified by Sarkosyl

rinsing and restarted by adjusting the elongation reactions to 7 mM Mg^{2+} , 1 mM ATP, GTP, and CTP, and 20 μ M UTP. Transcript elongation on the pML5-4NR template at suboptimal UTP concentrations was essentially blocked at the first intrinsic termination site (+195), even when the reaction was continued for 20 min (Fig. 1B, lanes 1,3–5). A reaction run for 5 min at 20 μ M UTP and then supplemented with UTP to 1 mM, followed by an additional 3-min incubation (lane 2), resulted in ~15% of the polymerases becoming blocked in elongation at each of the intrinsic termination sites, as we reported previously using similar conditions (Izban and Luse 1991). The addition of SII before elongation for 10

min (lane 6), or elongation for 10 min followed by addition of SII and a subsequent 5-min incubation (lane 7), resulted in the production of a significant number of transcripts that were clearly shorter than the RNAs generated by elongation blockage in the absence of SII (lane 3). There appeared to be too many of these shorter RNAs in the reactions containing SII to be accounted for by facilitated chase of the RNA associated with the complexes originally blocked at +135 and +147. Furthermore, the amount of transcript present at the original site of elongation blockage was reduced after exposure to SII (additional data not shown). Thus, we were forced to consider the possibility that the ternary transcription complex can catalyze the cleavage of the nascent transcript in the presence of SII. It is important to note that some of the transcripts appeared to be truncated by >15 bases. Because all transcripts are labeled only within the first 15 nucleotides, any cleavage of the RNA must have occurred from the 3' end. We presumed that our ability to detect shortened transcripts in reactions containing suboptimal concentrations of UTP resulted from successful competition of cleavage against the greatly reduced elongation rate.

Cleavage of nascent transcript within artificially paused RNA polymerase II ternary complexes requires Mg^{2+} and is greatly facilitated by SII

To test the effects of SII in a definitive way, we used a homogeneous population of paused RNA polymerase II ternary complexes containing 20-nucleotide transcripts. The complexes were generated using a derivative of the Ad2 ML promoter in which the initial transcribed region has the following sequence: 5'-ACUCUCUCCCCU-UCGCUUUAAAGC...-3'. Dinucleotide-primed initiation using 2 mM ApC, 10 μ M dATP, 10 μ M UTP, and 0.5 μ M [32 P]CTP generates ternary complexes paused predominantly after the synthesis of 14- and 15-nucleotide transcripts (data not shown). These complexes are then chased to the A-stop at +20 with 10 μ M CTP and GTP and purified by Sarkosyl rinsing as indicated previously. In this report we refer to artificially paused RNA polymerase II ternary complexes by the last base in the transcript and the position of that particular nucleotide. Therefore, the ternary complexes just described are termed U20 complex (note the underlined U preceding the run of A residues in the transcript sequence). In practice, RNA from U20 complex contained trace amounts of 21-, 22-, and 24-nucleotide RNAs (Fig. 2A, lane 1). The majority of these complexes are competent to resume transcription with the addition of Mg^{2+} and NTPs (see below).

Incubation of U20 complex with Mg^{2+} and 1 unit (Fig. 2A, lanes 2–7) or 0.5 unit (lane 9) of SII resulted in a rapid and extensive truncation of the nascent transcript. Remarkably, the predominant products of the truncation reaction are transcripts shortened in increments of 2 bases (additional data presented below). The truncation requires Mg^{2+} (lane 1) and is greatly reduced without SII after 5 (lane 10) or even 30 (lane 12) min of incubation.

The truncated RNAs remained in active ternary complexes and contained 3'-OH termini, as nearly all of them could be elongated when 1 mM NTPs were added (lane 7). Furthermore, the mobilities of purified shortened transcripts were not altered by phosphatase treatment and were identical to those of the corresponding transcripts generated by stalling elongation complexes using reaction conditions with limiting nucleotide concentrations (data not shown), which also supports the notion that truncation generates 3'-OH termini on the RNA remaining in ternary complex. RNA polymerase II must be involved in truncation, as this process is inhibited at α -amanitin concentrations that also inhibit chain elongation by RNA polymerase II (lane 8). A comparison of the cleavage products generated in 5 min with varying amounts of SII demonstrates that truncation is dependent on SII concentration. The 5-min reaction containing 1 unit of SII generated predominantly 10-nucleotide transcripts (lane 6), whereas the reaction containing 0.5 unit generated primarily 14-nucleotide products (lane 9). This is consistent with the observed concentration dependence of SII-mediated readthrough at intrinsic termination sites (SivaRaman et al. 1990; Bengal et al. 1991). Originally, we suspected that the limited transcript cleavage observed with nonsupplemented complexes reflected an inherent activity of the ternary complex. Subsequently, however, we have obtained indirect evidence that our Sarkosyl-rinsing procedure may not remove all elongation factors; this point will be considered in more detail below.

We have shown that transcription in the presence of 100 mM NH_4Cl greatly reduces the block to elongation at intrinsic termination sites (Izban and Luse 1991). Addition of NH_4^+ to RNA polymerase II halted at the intrinsic termination sites shown in Figure 1 resulted in the release of a small but significant portion of the blocked complexes into productive elongation (data not shown). Furthermore, transcription in the presence of NH_4^+ ions increases the rate at which RNA polymerases elongate transcripts (Sluder et al. 1988; Izban and Luse 1991). Incubation of U20 complex in the presence of Mg^{2+} and NH_4^+ for 5 or 30 min (Fig. 2A, lanes 11,13) resulted in less truncation than with Mg^{2+} alone (lanes 10,12). Also, the complexes tended to truncate by only 1 nucleotide with NH_4^+ . The preference for single-nucleotide transcript cleavage in the presence of NH_4^+ was observed consistently with all complexes tested (see additional data below).

A previous study indicated that the stimulatory activity of SII in a dC-tailed template assay is essentially identical in reactions containing either Mg^{2+} or Mn^{2+} (Reinberg and Roeder 1987), although RNA polymerase II activity is higher in nonspecific transcription assays performed with Mn^{2+} (Roeder 1976). When we performed transcript cleavage for 1 or 5 min after the addition of SII and 7 mM Mn^{2+} (Fig. 2B, lanes 2,3), we found that the kinetics and the apparent dinucleotide preference for transcript cleavage were altered. Elongation factor TFIIX is also capable of reducing the block to elongation at intrinsic termination sites when added either before or

Figure 2. Cleavage of nascent transcript in the U20 complex is facilitated by elongation factor SII. Lengths of RNAs are indicated at left in A and B. (A) U20 complex was incubated for the times given in the caption after the addition of SII (0.5 or 1 unit), Mg^{2+} , or NH_4^+ , as indicated. The time-course experiment (lanes 2–7) was performed by removing aliquots from a single large reaction at the specified times. The elongation competency of ternary complexes after 5 min was tested by supplying 1 mM NTPs to the last aliquot and incubating for 30 sec before stopping the reaction (lane 7). The reaction in lane 8 was preincubated with α -amanitin (1 $\mu\text{g}/\text{ml}$) for 3 min before adding SII and Mg^{2+} . (B) U20 complex was incubated for the indicated times after the addition of Mg^{2+} , Mn^{2+} , SII (0.1 or 1 unit), or TFIIX, as shown. RNA products were resolved on short (12.5 cm) 25% acrylamide/3% bisacrylamide sequencing gels.

after the arrival of RNA polymerases at such sites (Bengal et al. 1991). When U20 complexes were incubated with Mg^{2+} and TFIIX for 1 or 5 min (Fig. 2B, lanes 4,5), the nascent transcript was cleaved in a manner similar to that seen in SII-supplemented reactions. The ability of TFIIX to mediate the truncation reaction appears to be reduced compared with that observed by SII; however, as TFIIX is only partially pure, it is not possible to compare meaningfully the extent of truncation supported by each factor. Although the elongation rate stimulatory activity of the TFIIX fraction is probably not the result of TFIIF contamination (Bengal et al. 1991), it is not known whether the ability of this fraction to decrease pausing or facilitate transcript truncation results from the presence of SII or SII-related factors (Kanai et al. 1991) in the TFIIX preparation. Further purification of this fraction should resolve this issue.

Prompted by the observed decrease in the rate of transcript cleavage at lower SII concentrations, we performed a time course with limiting SII in an attempt to illustrate further the preference for dinucleotide cleavage. Tran-

scripts from reactions containing 0.1 unit of SII were stopped after 0.25, 1, or 5 min (Fig. 2B, lanes 6–8). The predominant cleavage product after 0.25- and 1-min incubations was 18 nucleotides long, with trace levels of 16-, 14-, and 12-nucleotide RNAs. The 5-min reaction contained predominantly 14- and 12-nucleotide transcripts. Transcripts of 19 and 17 nucleotides were only generated after 5 min of incubation; we attribute these RNAs to transcript cleavage of A21 complex. All of these data strongly suggest that cleavage occurs in dinucleotide increments. We have not proven this point, as we have not recovered the RNAs that are liberated from the complexes by cleavage. Single and dinucleotide cleavage products would have run off the end of the gels that we used. If, however, cleavage had occurred by the removal of larger fragments, we would have observed the RNAs released from the 3' end once truncation had proceeded into the portion of the transcript that is labeled (the first 15 nucleotides). For example, if the prominent 12-nucleotide transcripts in lanes 5 and 8 of Figure 2B had been produced by a single cleavage, we would have seen a

labeled RNA of 8 nucleotides produced. It should also be noted that we cannot rule out the possibility that cleavage occurs 1 nucleotide at a time, with every other cleavage being strongly rate limiting.

It is well established that transcripts may be cleaved within RNA polymerase ternary complexes by pyrophosphorolysis, an enzyme-catalyzed reaction in which the 3'-terminal NMP is removed with addition of pyrophosphate to form the free NTP (see Krummel and Chamberlin 1989 and references therein; Metzger et al. 1989). Thus, a pyrophosphate contaminant in the SII preparation might be responsible for the results that we observe. This seems unlikely, given the fact that the SII has been highly purified. To address this question directly, however, we incubated U20 and C15/U18 (see below) complexes with various concentrations of pyrophosphate. Transcript cleavage with either complex after 0.25 min with SII was more extensive than in a 10-min (U20 complex) or 1-min (C15/U18 complex) reaction containing 0.1 mM pyrophosphate (data not shown). Furthermore, pyrophosphate induced cleavage primarily in single-nucleotide increments. We conclude that pyrophosphorolysis is not involved in SII-facilitated transcript cleavage.

Transcripts may be cleaved down to only 4 nucleotides; evidence that the catalytic site of RNA polymerase II is repositioned to the proper site on the template after transcript truncation

Given that incubation of U20 complex with SII for only 15 sec led to measurable production of 10-nucleotide RNA (Fig. 2A, lane 2), we found it surprising that very little RNA shorter than 10 nucleotides had accumulated after a 5-min incubation (lane 6). This suggested that the complex bearing a 10-nucleotide transcript was at least an unusually stable intermediate. We have shown that RNA polymerase II initiated from the Ad2 ML promoter does not clear the promoter and become elongation committed until a transcript of 8–10 nucleotides is made (Cai and Luse 1987; Jacob et al. 1991). Thus, the reduction in the rate of cleavage when ternary complexes contain 10-nucleotide transcripts could reflect an important structural transition in the pathway to a stable elongation complex. We therefore attempted to generate complexes containing shorter transcripts by extending the reaction time. Incubation of U20 complex with SII for 30 min generated transcripts of 10, 9, 8, 6, and 4 nucleotides (Fig. 3A, lane 6). Transcripts shorter than 4 nucleotides would contain no labeled CTP and thus could not be detected. Because transcript cleavage requires that nascent RNA remain in ternary complex (see below), these data indicate that it was possible to generate ternary complexes containing as little as 4 nucleotides. However, cleavage of transcripts shorter than 9 nucleotides was not favored, as ~30% of RNAs were 9 or 10 nucleotides long even after 30 min of incubation. The majority of the 10-nucleotide and approximately half of the 9-nucleotide transcripts were chased with the addition of NTPs, whereas transcripts shortened to 8 nucleotides or less were not elongated (lane 7). Thus, SII-facilitated transcript cleav-

age in the U20 complex leads to the formation of metastable complexes containing predominantly 10-nucleotide transcripts. Further cleavage either leads to complex instability or to an elongation-deficient complex. Control reactions in which U20 complex was incubated with Mg^{2+} alone for 30 min (lane 11) and subsequently chased (lane 12) demonstrated that the Sarkosyl-rinsed ternary complexes without added SII can still generate a small proportion of elongation-competent complexes containing transcripts from which as many as 10 nucleotides have been cleaved. Finally, incubation of ternary complexes with NH_4^+ for 5 min (lane 2) did not alter their capacity to resume transcription (lane 3).

To test whether the catalytic site of RNA polymerase II remains in register with the coding strand during transcript cleavage we incubated U20 complex with SII for 5 min (see Fig. 3A, lane 4) and then allowed transcription to resume in the presence of 100 μM CTP, GTP, and UTP (lane 8). If the catalytic site of RNA polymerase II remained poised on the DNA template at position +20 during transcript cleavage, subsequent elongation would not have occurred because the substrate for the next three polymerization reactions (ATP) was not provided. We found that the majority of cleaved transcripts were elongated to position 20 (lane 8), consistent with the repositioning of the catalytic site during cleavage such that transcript elongation is resumed at the proper place on the template strand. A significant number of complexes, however, with 16- to 18-nucleotide RNAs were also detected. These complexes could have been generated by catalytic sites that became out of register by a few nucleotides, thereby generating shorter transcripts, although elongation continued to the triple A-stop. We feel that this is unlikely because a similar distribution of these complexes was also generated in reactions where SII was added after the addition of 100 μM CTP, UTP, and GTP (lane 9). To demonstrate that the 16- and 18-nucleotide transcripts generated in lane 8 were not the result of out-of-register elongation, we took advantage of the Sarkosyl and high salt sensitivity of SII-mediated readthrough at intrinsic termination sites. We performed three reactions (Fig. 3B) that were identical to the reaction shown in Figure 3A, lane 8, except that lanes 2 and 3 were supplemented with Sarkosyl or KCl as indicated immediately after the addition of NTPs. Thus, in these latter two reactions where SII activity was suppressed, all complexes were expected to chase quantitatively to the triple A-stop. The results in lanes 2 and 3 show unequivocally that the catalytic site of RNA polymerase retreats in register during the truncation reaction. Furthermore, these data indicate that the 16- and 18-nucleotide transcripts in Figure 3B, lane 1, and Figure 3A, lanes 8 and 9, arose because the truncation reaction is capable of competing with the polymerization reaction. To further explore this possibility, U20 complex was extended to A23 complex by adding 100 μM ATP before the addition of SII and subsequent 5-min incubation (Fig. 3A, lane 10). The products generated in this reaction were essentially identical to those generated in our standard reaction containing SII but devoid of nucleotides (lane 4).

Figure 3. Extended cleavage of transcript in U20 complex; competition between SII-facilitated cleavage and transcript elongation. (A) In lanes 1–7, 11, and 12, U20 complex was supplemented with Mg²⁺, SII, or NH₄⁺ and incubated for the times indicated. The reactions in lanes 3, 5, 7, and 12 were subsequently chased for 30 sec after supplying NTPs to 1 mM. In lane 8, U20 complex was incubated with Mg²⁺ and SII for 5 min before a 1-min elongation with 100 μ M GTP, UTP, and CTP. In lanes 9 and 10, U20 complex was preincubated for 1 min after supplying Mg²⁺ and either GTP, UTP, and CTP to 100 μ M (lane 9) or ATP to 100 μ M (lane 10) before the addition of SII and subsequent 5-min incubation. (B) Lane 1 was performed exactly as in A, lane 8. The reaction in lanes 2 and 3 were performed as in lane 1 except that Sarkosyl (lane 2) or KCl (lane 3) was added to the concentrations indicated immediately after NTP addition. Transcripts were purified and resolved as in Fig. 2. Transcript sizes are indicated at left in A and B.

These results taken together indicate that after transcript cleavage, the catalytic site and the 3' end of the transcript are properly aligned on the coding strand and that transcript cleavage competes with polymerization, at least at the nucleotide concentrations tested here.

Transcript cleavage with C15/U18 complex; truncation requires that transcripts be in ternary complex

We then sought to determine whether early elongation complexes halted at positions other than +20 would show the same ability to cleave their nascent transcripts. We have described another variant of the Ad2 ML promoter, pML5A (Jacob et al. 1991), with an initial transcribed sequence of 5'-ACUCUCUCCCCUUCG-CUGUCUGCGUGGGCCUGCUAA...-3'. Use of this template allows the synthesis of transcripts paused predominantly at +15, before the addition of the first G

residue (Jacob et al. 1991). In practice, we find that it is difficult to avoid significant leak-through to the second G-stop at +18; thus, transcription of pML5A in the absence of GTP produces a C15/U18 complex (underlined in the sequence above; Fig. 4A, lane 1). Supplementation of C15/U18 complex with Mg²⁺ and SII followed by 0.25 (lane 7) or 5 (lane 8) min of incubation resulted in the accumulation of shortened transcripts. The smallest transcript generated in 5 min was 9 nucleotides, 1 base shorter than the smallest RNA produced with 5-min incubations of U20 complex. The complex containing the 9-nucleotide RNA was also the predominant product observed after 30 min of incubation with SII (data not shown). As with the U20 complex, all of the transcripts (with the exception of a small fraction corresponding to C9) remained in elongation-competent ternary complexes (lane 9). Approximately half of the 9-nucleotide transcripts and all of the more extensively truncated

Figure 4. The effects of SII, NH₄⁺ ions, and α -amanitin on C15/U18 complex transcript cleavage; transcripts must remain in ternary complex to be truncated. Transcript sizes are noted at *left* in A and B. Transcripts were purified and fractionated as described in Fig. 2. (A) C15/U18 complex was supplemented with Mg²⁺ and SII, as indicated, and incubated for the times shown. Reactions (lanes 4,6,9) were subsequently chased for 30 sec after supplying NTPs to 1 mM. (B) Transcripts from C15/U18 complex were purified and resuspended in 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, at 30 times the concentration of a typical transcript cleavage reaction. Purified transcript (1 μ l) was added to 30 μ l of either reaction buffer (lanes 1–3) or C15/U18 complex generated with nonlabeled nucleotides (lanes 4,5) and incubated for 5 min after the

addition of Mg²⁺ or SII, as indicated. C15/U18 complex was incubated for the times shown after the inclusion of Mg²⁺ and NH₄⁺ as indicated (lanes 6–11), except that the reaction in lane 11 was preincubated with α -amanitin (1 μ g/ml) before the addition of Mg²⁺.

RNAs were not elongated after the 30-min reaction (data not shown). The dinucleotide increment of cleavage was not as obvious with C15/U18 complex, probably because the two complexes are paused 3 nucleotides apart. Note, however, that after a 15-sec incubation with SII (lane 7), the predominant transcript sizes below U18 and C15 were C16 and U13, respectively. No detectable cleavage occurred after a 5-min incubation in the absence of Mg²⁺ (lane 1). The behavior of C15/U18 complex supplemented with Mg²⁺ and incubated for 5 (lanes 2,5) or 30 (lane 3) min demonstrated more clearly than with U20 complex that the Sarkosyl-rinsed RNA polymerase II ternary complexes have a limited capacity to cleave nascent transcripts in reactions not supplemented with elongation factors; these shortened transcripts were retained in elongation-competent ternary complexes (lanes 4 and 6). We have shown that a small proportion of the RNA polymerases that are halted within intrinsic termination sites restart in reactions devoid of SII (Izban and Luse 1992). This could reflect an inherent but limited capacity of the RNA polymerase II ternary complex to truncate its transcript. Alternatively, our Sarkosyl-rinsed complexes could contain low levels of SII or an SII-like activity. We favor the latter hypothesis because the residual transcript cleavage activity of the rinsed complexes was sensitive to either 0.3% Sarkosyl or 250 mM KCl (data not shown). The elongation competency of the paused complexes deteriorated during the incubation in Sarkosyl at 37°C; within 5 min no resumption of elon-

gation was observed with NTP addition. The elongation complexes treated with high salt, however, remained active. Consistent with our interpretation, paused elongation complexes generated on dC-tailed templates with purified RNA polymerase II do not exhibit cleavage activity in the presence of Mg²⁺ (M. Chamberlin, pers. comm.).

We presumed that RNA must be in ternary complex to be cleaved because α -amanitin inhibits transcript cleavage. To demonstrate this directly, we purified radiolabeled transcripts from C15/U18 complex (Fig. 4B, lane 1) and tested for cleavage after incubation with Mg²⁺ (lane 2) or Mg²⁺ and SII (lane 3). We also incubated labeled C15/U18 transcripts with nonlabeled Sarkosyl-rinsed C15/U18 complex supplemented with either Mg²⁺ (lane 4) or Mg²⁺ and SII (lane 5). In no case was transcript cleavage observed. In addition, we investigated the effect of NH₄⁺ on transcript cleavage by the C15/U18 complex. Sarkosyl-rinsed C15/U18 complex was incubated for 5 (lanes 7,8) or 30 (lanes 9,10) min after the addition of Mg²⁺ (lanes 7–10) and NH₄⁺ (lanes 8,10). These data indicated more clearly that transcript cleavage in the presence of NH₄⁺ favors cleavage by a single nucleotide. In addition, we demonstrated that transcript cleavage that occurs without added SII is inhibited almost completely by α -amanitin (cf. lanes 11 and 9). Although a low level of transcript cleavage did occur in the reaction containing α -amanitin, we observed a comparable level of transcript elongation in the presence of high levels of

NTPs and α -amanitin (data not shown here, but see Linn and Luse 1991). These results demonstrate directly that RNA polymerase II participates in transcript cleavage.

Transcript cleavage of U35 complex generates predominantly C31 complex

The pML5A template also allows one to synthesize a ternary complex bearing predominantly 35-nucleotide RNAs (U35 complex) by initiating with a dinucleotide primer and transcribing in the absence of ATP (see pML5A initial transcribed sequence above, and Materials and methods). A 5-min time course of SII-facilitated transcript cleavage of U35 complex shows that predominantly C31 complex is formed (Fig. 5, lanes 2–6). Incubating U35 complex with 1 (lane 6) or 2 (lane 9) units of SII for 5 min also yielded detectable levels of transcripts of 27, 25, 23, 21, 17, 12, and 10 nucleotides. For comparison, a 5-min transcript cleavage reaction using C15/U18 complex (lane 10) and a subsequent elongation (lane 11) are shown. As was the case with the other complexes, the RNAs generated by transcript cleavage of U35 quantitatively chased upon addition of 1 mM NTPs (lanes 7,8). Although the U35 complex supported less extensive transcript cleavage compared with complexes halted earlier in elongation, a small proportion of U35 RNAs was truncated by 25 nucleotides, down to 10-nucleotide RNAs. The pattern of U35 cleavage products smaller than 18 nucleotides was similar to the pattern observed with U20 and U18 complexes (cf. Fig. 5, lane 9 to lane 10, and Fig. 2A, lane 6). Because the first 20 nucleotides of transcript in all three complexes were identical, we suspected that transcript sequence might influence the progress of the cleavage reaction.

Transcript cleavage of paused RNA polymerase II ternary complexes generated from a mouse β -globin promoter

To investigate further the influence of transcript sequence on cleavage, we generated RNA polymerase II ternary complexes halted after the synthesis of 15 and 21 nucleotides using templates pMB20 and pMB5T, respectively. Each plasmid contains a modified version of the mouse β -globin (MB) promoter. The sequences of the initial transcribed regions are

pMB20, 5'-ACUUUUCCUUCUGGCAAA...
pMB5T, 5'-ACUUUUCCUUCUGGCGGCCGCAA...

where C15 and C21 are underlined. C15 and C21 complexes were obtained by transcription of these two templates in the absence of ATP (Fig. 6, lanes 1,9); the C15 complex also contained a significant amount of A16 complex (Fig. 6, lane 1). Both complexes showed a very limited capacity for transcript cleavage in the absence of

Figure 5. SII-facilitated cleavage of nascent RNA in U35 complex. U35 or C15/U18 complex was supplemented with Mg²⁺ and SII (1 or 2 units) and incubated for the times indicated. Reactions (lanes 7,8,11) were subsequently chased for 30 sec after supplying NTPs to 1 mM. Transcripts were resolved on a 15% (29:1 acrylamide/bisacrylamide) sequencing gel. Transcript sizes are indicated at left.

SII. SII-facilitated transcript cleavage with the C15 complex generated predominantly 11-nucleotide transcripts with trace levels of 14-, 10-, and 9-nucleotide RNAs after a 5-min cleavage reaction (lanes 6,7). All of these transcripts were retained in elongation-competent ternary complexes (lane 8). These products differ from those generated with the Ad2 ML C15/U18 complex (see Fig. 4A, lane 8). A 5-min SII-facilitated transcript cleavage reaction with the C21 complex generated shorter RNAs (15, 14, and 11 nucleotides) that were essentially identical to

SII-dependent transcript truncation

Figure 6. Transcript cleavage within C15 and C21 complexes generated with the MB promoter. C15 or C21 complex was supplemented with Mg²⁺ or SII and incubated for the times indicated. Reactions (lanes 3,5,8,11,17) were subsequently chased for 30 sec after supplying NTPs to 1 mM. Transcripts were resolved as described in Fig. 2. Transcripts from an entire reaction were used, however, because the MB promoter is less efficient at initiation than the Ad2 ML promoter. Transcript sizes are indicated in the center.

those obtained with C15 complex (cf. lanes 7 and 16). All of the complexes with 11- to 15-nucleotide RNAs were fully elongation competent (lane 17). In the early stages of the cleavage reaction with C21 complex, the predominant product was an 18-nucleotide RNA (lanes 12,13). C18 remained the most abundant complex produced after a 5-min reaction (lane 16; note that the lengths of the RNAs in Figure 6 were confirmed by electrophoresis on much longer gels, not shown, in the presence of appropriate markers). The production of the C18 complex from C21 is the most prominent deviation we have seen to date from the rule that SII-mediated cleavage occurs in dinucleotide increments. The apparent stability of the C18 complex may be important in this regard; that is, production of an unusually stable intermediate may be sufficiently favored during transcript truncation that the preferred cleavage increment may be violated. The predominant cleavage products formed with C21 complex in 5-min reactions differed considerably from those generated under the same conditions with the Ad2 ML U20 complex (primarily 10- and 12-nucleotide RNAs; see Fig. 2A, lane 6). Therefore, transcript cleavage may also be influenced by transcript or template sequence, or both.

Discussion

The control of gene expression at the level of transcript elongation is now well established (for review, see Spencer and Groudine 1990). The importance of understanding this regulatory mechanism is emphasized by its use in controlling the expression of a number of genes, such as *c-myc* and *c-fos*, whose products are regulators themselves. To describe fully the molecular mechanisms involved in elongation control within particular genes, it

will be necessary to understand in much greater detail both the basic process of transcript extension by RNA polymerase II and the influence that general elongation factors and DNA template configuration have on this process. We have begun to explore these questions using both pure DNA and chromatin templates as substrates for transcription (Izban and Luse 1991, 1992). In the course of our studies, we discovered that RNA polymerase II blocked in elongation at intrinsic termination sites can cleave its nascent transcript in the 3' → 5' direction in the presence of elongation factor SII. This activity was initially detected at intrinsic termination sites during elongation at suboptimal nucleotide concentrations. The SII-facilitated nuclease activity, however, appears to be a general property of ternary complexes, as a variety of complexes generated from two different promoters and halted artificially at discrete positions early in elongation also exhibit this activity, both in reactions devoid of NTPs and in those with limiting (100 μM) NTP concentration.

It has been observed recently that certain *Escherichia coli* RNA polymerase ternary complexes halted early in elongation by NTP limitation can cleave their nascent transcripts spontaneously in a Mg²⁺-dependent reaction (Surratt et al. 1991). The 5' portions of these transcripts remain in ternary complex and can be elongated upon addition of NTPs. Complexes paused after the synthesis of 7-, 8-, 20-, and 21-nucleotide RNAs liberate 2-, 3-, 10-, and 10-nucleotide fragments, respectively, but either no or slow cleavage was observed for complexes paused at other positions. This is clearly a different phenomenon from that observed with RNA polymerase II. Cleavage in the RNA polymerase II ternary complex is essentially dependent on an additional factor. All of the eukaryotic

ternary complexes were highly active in transcript cleavage in the presence of SII and the absence of NTPs. Furthermore, nearly all of the complexes appeared to truncate the nascent RNA primarily in dinucleotide increments. We saw no evidence for the release of longer RNA fragments.

While this work was being prepared for publication, Reines (1992) reported a similar effect of SII using RNA polymerase II ternary complexes halted at a well-characterized intrinsic terminator of the human histone gene in reactions devoid of NTPs. These elongation complexes were initiated with ATP and purified by a different approach than we used here. D. Reines notes (1992) that recombinant murine SII will also mediate transcript cleavage. These findings reinforce the general nature of the process and argue against the possibility that the cleavage we observe is dependent on the use of a dinucleotide primer during initiation, on our particular method of transcription complex purification, or on a contaminant in the SII preparation. Both studies leave open the issue of the intrinsic ability of polymerase II ternary complex to cleave its nascent RNA. On the basis of earlier work (Hawley and Roeder 1985; Reines et al. 1989), we had expected that exposure to Sarkosyl would remove SII quantitatively. As noted above, however, the addition of 250 mM KCl to the ternary complexes completely abolishes any residual ability to truncate transcripts without affecting their activity in chain elongation. Thus, we now suppose that the low level of cleavage activity in the Sarkosyl-rinsed complexes results from the presence of a low level of either SII or an SII-like activity in these preparations. The exact nature of this activity is currently being investigated.

It seems counterintuitive that a factor that facilitates elongation should also mediate transcript cleavage by the elongation complex. Although we can only speculate on the cleavage mechanism, certain features of the truncation process are comparable to aspects of the proofreading activity of DNA polymerases (for review, see Echols and Goodman 1991). It is important to emphasize that we have no evidence that misincorporation of nucleotides facilitates SII-mediated transcript cleavage. There are mechanistic similarities, however, between transcript truncation and proofreading that may suggest an explanation for the paradoxical behavior of SII. Occupancy of the DNA polymerase exonuclease site by the newly replicated strand is favored after incorporation of a mismatched base pair. This is presumed to result from both pausing due to the misalignment of the 3'-OH within the catalytic site and destabilization of the hybrid. Moreover, during DNA synthesis, there is a low but measurable probability that correctly incorporated bases will still be excised by the exonuclease. SII-mediated transcript cleavage by the RNA polymerase II ternary complex is also a slow process that can only be readily observed with completely halted ternary complexes in the absence of NTPs or when elongation rates are very low, for example, at intrinsic termination sites with one NTP limiting. The possibility that RNA polymerase II might have nuclease activity was raised recently by Shi-

rai and Go (1991), who noted a similarity between the second largest RNA polymerase II subunit and a number of bacterial RNases. By analogy with DNA polymerase proofreading, we may presume that the block to elongation by RNA polymerase II within intrinsic termination sites might be the result of a misalignment of the 3' end of the transcript within the RNA polymerase. Thus, the role of SII-dependent transcript cleavage could be facilitating the removal of the 3' terminus of the transcript to reestablish appropriate alignment. Because the rate of SII-facilitated cleavage is only a small fraction of the rate of polymerization, competition between cleavage and transcript elongation in reactions containing optimal NTP concentrations may not be readily detected. Indeed, transcription rates of core RNA polymerase II do not appear to be affected by SII (Bengal et al. 1991; Izban and Luse 1992). Although a block to elongation is not a prerequisite for SII-facilitated cleavage (Fig. 3, lanes 8–10), complete blockage or elongation at greatly reduced rates would naturally favor the transcript cleavage reaction, as we observed. It is useful to note that a significant portion of core RNA polymerase II ternary complexes are not blocked in elongation during transcription through intrinsic termination sites (see Fig. 1B, lane 2). Consequently, SII may ultimately facilitate readthrough at these sites by allowing the polymerase to "back up" and retranscribe regions of DNA that induce blocks to elongation.

We have also shown that transcription by core RNA polymerase II is strongly inhibited on chromatin templates (Izban and Luse 1991). Our data suggest that pausing on chromatin templates is the result of the inability of polymerase to efficiently transcribe through a nucleoprotein structure (the nucleosome) and that the sites of transcriptional blockage are determined by the underlying DNA sequence. Interestingly, we have found that SII stimulates transcription through chromatin templates, although elongation remains much less efficient than on naked DNA (Izban and Luse 1992). Furthermore, comparative studies on the effects of all three elongation factors (TFIIF, TFIIX, and SII), either singly or in combination, led us to conclude that the rate-limiting step during transcription through nucleosomal templates was restarting paused polymerases. Therefore, SII may ultimately facilitate transcription on chromatin templates in a manner similar to that discussed for the intrinsic termination site, that is, by allowing the polymerase repeated attempts to transcribe through certain regions where blocks to elongation are preferred. This implies that in addition to regulating elongation at relatively rare intrinsic termination sites, the SII class of proteins may also have a more general role during the transcription process *in vivo*.

Our data indicate that the catalytic site on the template must reposition itself as a consequence of transcript truncation. We do not know whether contacts with the template surrounding the catalytic site are also altered. It is difficult, however, to imagine how U20 complex, for example, could truncate transcript by as much as 10 nucleotides without repositioning these con-

tacts. Interestingly, Metzger et al. (1989) have shown by exonuclease III footprint analyses of *E. coli* RNA polymerase positioned at various different sites that one particular C20 complex was not stable to such treatment. After digestion, a complex was formed that contained only an 11-nucleotide transcript. The transcript was shortened 3' → 5' and retained in ternary complex, although the elongation competency of the complex was not tested. Moreover, the resultant exonuclease III digestion pattern obtained was that expected for a complex containing an 11-mer, not a 20-mer. Thus, in this one case, RNA polymerase was shown to move back along the DNA template as a consequence of transcript truncation.

Certain features of the process of transcript cleavage by RNA polymerase II may help us to understand in much greater detail the general mechanism of transcript elongation in eukaryotic cells. In this context, it is useful to recall the changes in structural properties that *E. coli* RNA polymerase undergoes during initiation and early elongation. Straney and Crothers (1987) proposed that a stressed intermediate is formed during the last step of initiation where escape into productive elongation competes with abortive transcription (ejection of the transcript followed by reinitiation without enzyme release). In this model translocation away from the promoter occurs by an inchworm-like motion. The leading edge of the complex stretches forward before release and rebinding of contacts in the upstream region. Although this event may be unique during the transition from an initiation to an elongation complex, we can hypothesize that further stressed intermediates may form after the synthesis of successive 10-nucleotide stretches of transcript, as the polymerase releases and rebinds the upstream portion of the template. On the basis of comparisons of gel-shift mobilities and DNA-protection patterns of RNA polymerase ternary complexes containing 11- to 35-nucleotide-long transcripts, another structural transition does appear to take place during the synthesis of 20- to 24-nucleotide-long transcripts (Carpousis and Gralla 1985; Straney and Crothers 1985; Metzger et al. 1989). The possibility has been raised recently that the structure of prokaryotic elongation complex might be constantly changing during elongation (Surratt et al. 1991). A widely accepted model holds that the stability of the elongation complex depends on the presence of a 10- to 12-nucleotide RNA/DNA hybrid; this would also account for the instability of the very early elongation complex (see Yager and Von Hippel 1991 and references therein). Rice et al. (1991), however, have shown that transcripts can be cleaved with RNase to within 3 nucleotides of the 3'-terminal growing point in an RNA polymerase II transcription complex; some of these short fragments are retained in active ternary complex capable of continued elongation. These investigators favor a model based on earlier proposals (Kumar 1981 and references therein) in which the ternary complex contains only a 2- to 3-nucleotide DNA/RNA hybrid. In this case, most of the nascent RNA within the transcription complex does not interact with the template but with one or

more RNA-binding domains (see also Kerppola and Kane 1991). This model was also invoked to account for the very different cleavage potentials of the various prokaryotic ternary complexes mentioned previously (Surratt et al. 1991). To accommodate cleavage of 10-nucleotide fragments from complexes halted after the synthesis of 20- to 21-nucleotide transcripts, Surratt et al. proposed that polymerase contains two product-binding domains, each of which accommodate 8–10 nucleotides of transcript and are perhaps set at angles to one another. Transcript hydrolysis was viewed as the result of stresses imposed on certain phosphodiester bonds within some complexes that were in a particular conformation or that bound the RNA exceptionally tightly, or both. The possibility that polymerase contains two RNA product-binding sites is also consistent with physical studies of the prokaryotic ternary complex, which showed that fully stable "mature" *E. coli* ternary elongation complex may not be formed until after the synthesis of a 20- to 24-nucleotide transcript (Carpousis and Gralla 1985; Straney and Crothers 1985; Metzger et al. 1989).

Abortive initiation does occur at both the Ad2 ML and MB promoters (Luse and Jacob 1987; Jacob et al. 1991; D. Luse and J. Kitzmiller, in prep.), and the transition between initiation and stable elongation complex formation occurs only after the synthesis of 8–10 nucleotides (Cai and Luse 1987; Jacob et al. 1991; D. Luse and J. Kitzmiller, in prep.). Furthermore, ternary complexes halted in elongation after the synthesis of 15- or 35-nucleotide transcripts differ with respect to structure, as determined by mobility shift and the extent to which they protect the underlying DNA sequence (Linn and Luse 1991). The ability of SII to facilitate transcript cleavage, thereby backing up RNA polymerase II, has afforded us a unique opportunity to examine the properties of the ternary complex as it approaches potential structural transition points in the reverse direction. Interestingly, Ad2 ML U20 and C15/U18 complexes cleaved nascent RNAs back to predominantly 10-nucleotide (Fig. 3, lanes 4,6) and 9-nucleotide (Fig. 4, lane 8, and additional data not shown) transcripts, respectively. Extended transcript cleavage reactions with either Ad2 ML U20 (Fig. 3, lanes 6,7) or C15/U18 (data not shown) complexes generated complexes bearing shorter transcripts; however, these complexes were unstable or elongation deficient. Thus, the transition to elongation competency observed during initiation is also observed in the "back" reaction. These results emphasize that the length of the transcript is important for ternary complex stability, regardless of the previous history of the complex. Stabilizing interactions of the transcript with other components of the transcription machinery should therefore be crucial for ternary complex stability.

Although all of the complexes that we tested (except for the C21 complex on the MB promoter) appeared to cleave their transcripts primarily in dinucleotide increments, a number of the intermediates that we observed in the truncation process were unusually stable. These include the C31, C17, and C10 complexes for the Ad2 ML promoter and the C18 and C11 complexes for the MB

promoter. Such differences in stability could reflect, for example, different phases of filling product-binding sites and/or different phases in the translocation process along the template. Polymerases may retreat to a point with the locally most favorable contacts on the template or unusually strong interactions of the transcript and the RNA polymerase. It should be emphasized that whereas all of the transcript cleavage reactions tested showed important similarities, the details of the truncation process, particularly the kinetics, differed for each complex. This may, at least in part, be the result of sequence-specific aspects of the cleavage process itself, as suggested by the comparison of the truncation reaction in Ad ML C15 and MB C15 complexes. This point is emphasized further by a result from experiments discussed previously by Metzger et al. (1989). Two complexes, each halted after the synthesis of a 20-nucleotide transcript, were generated using identical promoters and initial transcribed regions that differed by a 9-nucleotide insert between positions +11 and +12. Only one of these complexes proved to be sensitive to exonuclease III digestion.

In summary, we have shown that in the presence of elongation factor SII, the RNA polymerase II ternary complex can serve as a nuclease, cleaving its nascent transcript from the 3' end. This process leaves the ternary complex intact and the remaining transcript can be subsequently elongated. Transcript truncation might be an obligatory part of the process by which SII restarts polymerases paused at potential termination sites. We expect that much more detailed studies of the cleavage reaction using a variety of complexes with different transcript sequences and lengths will provide considerable new insight into the mechanism of enzyme translocation and the molecular interactions that stabilize the RNA polymerase II transcription complex. Understanding these aspects of elongation will be crucial in elucidating the mechanism by which gene expression is controlled through blocks to elongation.

Materials and methods

Plasmid construction

pSmaF-1 contains 2450 bp of Ad2 DNA bearing the ML promoter cloned into pBR322 (Knezetic and Luse 1986). The construction of the Ad2 ML promoter-bearing plasmids pML5A (Jacob et al. 1991) and pML5-4NR (Izban and Luse 1991) have been described in detail elsewhere. pMB5T contains a 124-bp fragment bearing a modified form of the MB promoter region, beginning just upstream of the TATA box, which was cloned into *XmaI*/*EcoRI*-cleaved pUC-18 such that transcription proceeds toward the *EcoRI* site (D. Luse and J. Kitzmiller, in prep.). pML20 was constructed by replacing a *Bss*HII-*Bam*HI fragment of pML5A with a synthesized fragment that was identical except for the sequence from +18 to +23, which was modified from 5'-TGTCTG to 5'-TTTAAA (noncoding strand). These base substitutions generate a *DraI* restriction endonuclease cleavage site. pMB20 was constructed by first linearizing pML20 at the unique *EcoRI* site upstream of the ML promoter, filling in the 5' overhang with DNA polymerase Klenow fragment, and then performing a partial digestion with *DraI*. A PCR-generated fragment containing the promoter of pMB5T

from -43 to +15 was then ligated into the purified pML20 fragment that lacked the Ad2 ML promoter. pMB20 contains the MB promoter cloned in the same orientation as the Ad2 ML promoter in pML20. All plasmids were verified by sequencing.

Preinitiation and elongation complex formation and purification

RNA polymerase II preinitiation complexes were formed on Ad2 ML and MB promoter-bearing plasmids as described previously (Izban and Luse 1991). Briefly, 150- μ l reactions containing 52% HeLa cell nuclear extracts and 19 μ g/ml of circular plasmid DNA were incubated at 25°C for 5 min and chilled on ice, and the protein-DNA complexes were isolated by gel filtration on BioGel A-1.5m. The majority of the void volume (~160 μ l), which contains the protein-DNA complexes, was collected.

Stable elongation complexes paused on the pSmaF-1 and pML5-4NR templates were generated as described previously (Izban and Luse 1991). Essentially the same approach was used with the pML5A, pML20, pMB5T, and pMB20 templates. Basically, 100 μ l of the gel-filtered preinitiation complexes were incubated in a 125- μ l reaction containing 2 mM ApC (Sigma), 10 μ M UTP, 10 μ M dATP, and 0.5 μ M [α -³²P]CTP (800 Ci/mmol; New England Nuclear). All nonradioactive nucleotides used during initiation and elongation were from Pharmacia (FPLC purified). After 5 min at 25°C, the reactions were chased with 10 μ M UTP and CTP to generate C15/U18 complexes (with the pML5A template) or 10 μ M UTP, CTP, and GTP to generate U20 (pML20 template) or U35 (pML5A template) complexes. An identical protocol was used to generate both C15 and C21 complexes with the MB promoter-bearing templates except that the initiation reaction contained 2 mM ApC, 10 μ M UTP, 10 μ M dATP, 1 μ M [α -³²P]CTP, and 1 μ M GTP and the subsequent chase was performed with 10 μ M UTP, CTP, and GTP. Also as described previously, elongation complexes were purified by Sarkosyl rinsing, that is, gel filtration after a short incubation with Sarkosyl (Izban and Luse 1991). The column running buffer was 30 mM Tris-HCl (pH 7.9), 10 mM β -glycerophosphate, 62 mM KCl, 0.5 mM EDTA, and 1 mM DTT. The void volume fractions containing radioactivity were pooled and usually contained 250 μ l.

Transcript cleavage reaction

The pooled complexes were made 8 mM in MgCl₂ and then divided into 30- μ l aliquots. Cleavage reactions were preincubated at 37°C for $\geq$ 2 min before the addition of SII or NH₄Cl; reactions were run at 37°C for the times indicated in the figure legends. One microliter of purified SII (0.077 μ g protein) was used per reaction unless otherwise indicated. In the experiments shown in Figure 2A, lanes 8–13, and Figures 2B and 4B, lanes 7–11, the reactions were preincubated at 37°C before the addition of α -amanitin (Boehringer Mannheim), MgCl₂, MnCl₂, or NH₄Cl, as indicated. Elongations were performed by adding 0.11 volume (usually 3.7 μ l) of 10 mM NTPs, followed by a 0.5-min incubation at 37°C. The reactions in Figure 1 were stopped, and the transcripts were purified as described previously (Izban and Luse 1991). All other reactions were stopped by the addition of 70 μ l of ice-cold 5 mM EDTA (pH 8.0) and remained on ice until they were phenol/chloroform extracted. For the time-course experiments, large initial reactions were used so that 30- μ l aliquots could be removed at the times indicated. Reactions were either stopped or chased, as indicated above. Transcripts were purified by sequential phenol/chloroform (1 : 1) and chloroform extractions, and the aqueous phase was dried under vacuum. The pellets were resuspended in 4 μ l of

water and 8 μ l of formamide dye mix, boiled for 3 min, and quick chilled on ice. Half of each sample was resolved on a polyacrylamide gel, as indicated in the figure legends. In the cases where we used the MB promoter, which is weaker than the Ad2 ML promoter, the pellets were resuspended in half the volumes indicated above and the entire sample was loaded. After electrophoresis, the gels were exposed (usually for 18 hr) to Kodak X-AR film with a Lightning Plus intensifying screen. The percent of transcripts of a particular length was quantitated using a PhosphorImager system from Molecular Dynamics (Sunnyvale, CA), as described previously (Izban and Luse 1991). In those cases where transcripts had been truncated into the region that contains radiolabeled nucleotides, a correction was made for the number of residues removed.

Elongation factors

Elongation factor SII was a gift from R. Weinmann. This factor was purified to homogeneity from calf thymus as described previously, and the protein concentration was 0.077 mg/ml (Rappaport et al. 1987). We emphasize that this elongation factor is of very high purity. No detectable contaminants were observed in this preparation when 1.5 μ g of protein was resolved by SDS-PAGE and visualized by either Coomassie or India ink staining procedures; SII stains poorly using the silver staining method (Rappaport et al. 1987). TFIIX was isolated from HeLa cells and was a gift from D. Reinberg. TFIIX was purified through the heparin-Ultrogel (LKB) chromatographic step (Reinberg et al. 1987). The protein concentration of the fraction used was 2.0 mg/ml. TFIIX activity was not resolved, however, from TFIID activity in the preparation we obtained (data not shown). One unit of SII or TFIIX was defined as the amount of elongation factor required to reduce pausing at the Ad2 ML or pML5-4NR major pause sites to their minimum levels in the standard reaction volume.

Acknowledgments

We are very grateful to R. Weinmann and D. Reinberg for their generous gift of purified elongation factors. We also thank M. Chamberlin for thoughtful discussions and D. Reines for communicating results before publication. This research was supported by grant GM 29487 from the National Institutes of Health (NIH). M.G.I. was supported by NIH postdoctoral fellowship GM 14111.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Agarwal, K., K.H. Baek, C.J. Jeon, K. Miyamoto, A. Ueno, and H.S. Yoon. 1991. Stimulation of transcript elongation requires both the zinc finger and RNA polymerase-II binding domains of human TFIIS. *Biochemistry* **30**: 7842–7851.
- Bengal, E., O. Flores, A. Krauskopf, D. Reinberg, and Y. Aloni. 1991. Role of the mammalian transcription factors IIF, IIS, and IIX during elongation by RNA polymerase II. *Mol. Cell. Biol.* **11**: 1195–1206.
- Cai, H. and D.S. Luse. 1987. Transcription initiation by RNA polymerase II in vitro. Properties of preinitiation, initiation and elongation complexes. *J. Biol. Chem.* **262**: 298–304.
- Carpousis, A.J. and J.D. Gralla. 1985. Interaction of RNA polymerase with lacUV5 promoter DNA during mRNA initiation and elongation. Footprinting, methylation, and rifampicin-sensitivity changes accompanying transcription initiation. *J. Mol. Biol.* **183**: 165–177.
- Echols, H. and M.F. Goodman. 1991. Fidelity mechanisms in DNA replication. *Annu. Rev. Biochem.* **60**: 447–511.
- Hawley, D.K. and R.G. Roeder. 1985. Separation and partial characterization of three functional steps in transcription initiation by human RNA polymerase II. *J. Biol. Chem.* **260**: 8163–8172.
- Hubert, J.-C., A. Guyonvarch, B. Kammerer, F. Exinger, P. Liljelund, and F. Lacroute. 1983. Complete sequence of a eukaryotic regulatory gene. *EMBO J.* **2**: 2071–2073.
- Izban, M.G. and D.S. Luse. 1991. Transcription on nucleosomal templates by RNA polymerase II in vitro: Inhibition of elongation with enhancement of sequence-specific pausing. *Genes & Dev.* **5**: 683–696.
- . 1992. Factor-stimulated RNA polymerase II transcribes at physiological rates on naked DNA but very poorly on chromatin templates. *J. Biol. Chem.* **267** (in press).
- Jacob, G.A., S.W. Luse, and D.S. Luse. 1991. Abortive initiation is increased only for the weakest members of a set of down mutants of the adenovirus 2 major late promoter. *J. Biol. Chem.* **266**: 22537–22544.
- Kanai, A., T. Kuzuhara, K. Sekimizu, and S. Natori. 1991. Heterogeneity and tissue-specific expression of eukaryotic transcription factor S-II-related protein mRNA. *J. Biochem.* **109**: 674–677.
- Kerppola, T.K. and C.M. Kane. 1990. Analysis of the signals for transcription termination by purified RNA polymerase II. *Biochemistry* **29**: 269–278.
- . 1991. RNA polymerase: Regulation of transcript elongation and termination. *FASEB J.* **5**: 2833–2842.
- Knezetic, J.A. and D.S. Luse. 1986. The presence of nucleosomes on a DNA template prevents initiation by RNA polymerase II in vitro. *Cell* **45**: 95–104.
- Krummel, B. and M.J. Chamberlin. 1989. RNA chain initiation by *Escherichia coli* RNA polymerase. Structural transitions of the enzyme in early ternary complexes. *Biochemistry* **28**: 7829–7842.
- Kumar, S.A. 1981. The structure and mechanism of action of bacterial DNA-dependent RNA polymerase. *Prog. Biophys. Mol. Biol.* **38**: 165–210.
- Linn, S.C. and D.S. Luse. 1991. RNA polymerase II elongation complexes paused after the synthesis of 15- or 35-base transcripts have different structures. *Mol. Cell. Biol.* **11**: 1508–1522.
- Luse, D.S. and G.A. Jacob. 1987. Abortive initiation by RNA polymerase II in vitro at the Adenovirus 2 major late promoter. *J. Biol. Chem.* **262**: 14990–14997.
- Maderious, A. and S. Chen Kiang. 1984. Pausing and premature termination of human RNA polymerase II during transcription of adenovirus in vivo and in vitro. *Proc. Natl. Acad. Sci.* **81**: 5931–5935.
- Metzger, W., P. Schickor, and H. Heumann. 1989. A cinematographic view of *Escherichia coli* RNA polymerase translocation. *EMBO J.* **8**: 2745–2754.
- Price, D.H., A.E. Sluder, and A.L. Greenleaf. 1989. Dynamic interaction between a *Drosophila* transcription factor and RNA polymerase II. *Mol. Cell. Biol.* **9**: 1465–1475.
- Rappaport, J., D. Reinberg, R. Zandomeni, and R. Weinmann. 1987. Purification and functional characterization of transcription factor SII from calf thymus: Role in RNA polymerase II elongation. *J. Biol. Chem.* **262**: 5227–5232.
- Reinberg, D. and R.G. Roeder. 1987. Factors involved in specific transcription by mammalian RNA polymerase II. Transcription factor IIS stimulates elongation of RNA chains. *J. Biol.*

- Chem.* **262**: 3331–3337.
- Reinberg, D., M. Horikoshi, and R.G. Roeder. 1987. Factors involved in specific transcription in mammalian RNA polymerase II. Functional analysis of initiation factors IIA and IID and identification of a new factor operating at sequences downstream of the initiation site. *J. Biol. Chem.* **262**: 3322–3330.
- Reines, D. 1992. Elongation factor-dependent transcript shortening by template-engaged RNA polymerase II. *J. Biol. Chem.* **267**: 3795–3800.
- Reines, D., M.J. Chamberlin, and C.M. Kane. 1989. Transcription elongation factor SII (TFIIS) enables RNA polymerase II to elongate through a block to transcription in a human gene *in vitro*. *J. Biol. Chem.* **264**: 10799–10809.
- Rice, G.A., C.M. Kane, and M.J. Chamberlin. 1991. Footprinting analysis of mammalian RNA polymerase II along its transcript: An alternative view of transcription elongation. *Proc. Natl. Acad. Sci.* **88**: 4245–4249.
- Roeder, R.G. 1976. Eukaryotic nuclear RNA polymerases. In *RNA polymerase* (ed. R. Losick and M. Chamberlin), pp. 285–329. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Shirai, T. and M. Go. 1991. RNase-like domain in DNA-directed RNA polymerase-II. *Proc. Natl. Acad. Sci.* **88**: 9056–9060.
- SivaRaman, L., D. Reines, and C.M. Kane. 1990. Purified elongation factor SII is sufficient to promote read-through by purified RNA polymerase II at specific termination sites in the human histone H3.3 gene. *J. Biol. Chem.* **265**: 14554–14560.
- Sluder, A.E., D.H. Price, and A.L. Greenleaf. 1988. Elongation by *Drosophila* RNA polymerase II. Transcription of 3'-extended DNA templates. *J. Biol. Chem.* **263**: 9917–9925.
- Sluder, A.E., A.L. Greenleaf, and D.H. Price. 1989. Properties of a *Drosophila* RNA polymerase II elongation factor. *J. Biol. Chem.* **264**: 8963–8969.
- Spencer, C.A. and M. Groudine. 1990. Transcription elongation and eukaryotic gene regulation. *Oncogene* **5**: 777–786.
- Straney, D.C. and D.M. Crothers. 1985. Intermediates in transcription initiation from the *E. coli* lac UV5 promoter. *Cell* **43**: 449–459.
- . 1987. A stressed intermediate in the formation of stably initiated RNA chains at the *Escherichia coli* lac UV5 promoter. *J. Mol. Biol.* **193**: 267–278.
- Surratt, C.K., S.C. Milan, and M.J. Chamberlin. 1991. Spontaneous cleavage of RNA in ternary complexes of *Escherichia coli* RNA polymerase and its significance for the mechanism of transcription. *Proc. Natl. Acad. Sci.* **88**: 7983–7987.
- Wiest, D.K. and D.K. Hawley. 1990. *In vitro* analysis of a transcription termination site for RNA polymerase II. *Mol. Cell. Biol.* **10**: 5782–5795.
- Yager, T.D. and P.H. Von Hippel. 1991. A thermodynamic analysis of RNA transcript elongation and termination in *Escherichia coli*. *Biochemistry* **30**: 1097–1118.

The RNA polymerase II ternary complex cleaves the nascent transcript in a 3'----5' direction in the presence of elongation factor SII.

M G Izban and D S Luse

Genes Dev. 1992, **6**:

Access the most recent version at doi:[10.1101/gad.6.7.1342](https://doi.org/10.1101/gad.6.7.1342)

References

This article cites 36 articles, 21 of which can be accessed free at:
<http://genesdev.cshlp.org/content/6/7/1342.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).