NIH Public Access
Author Manuscript
*J Biol Chem*. Author manuscript; available in PMC 2012 June 11.

Published in final edited form as:
*J Biol Chem*. 1992 August 5; 267(22): 15516–15522.

# The RNA Polymerase II Elongation Complex:

## FACTOR-DEPENDENT TRANSCRIPTION ELONGATION INVOLVES NASCENT RNA CLEAVAGE*

**Daniel Reines**[‡,§,¶], **Pejman Ghanouni**[§], **Qing-qin Li**[‡], and **John Mote Jr.**[§]

[‡]Graduate Program in Biochemistry and Molecular Biology, Atlanta, Georgia 30322

[§]Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322

## Abstract

Regulation of transcription elongation is an important mechanism in controlling eukaryotic gene expression. SII is an RNA polymerase II-binding protein that stimulates transcription elongation and also activates nascent transcript cleavage by RNA polymerase II in elongation complexes *in vitro*. Here we show that SII-dependent *in vitro* transcription through an arrest site in a human gene is preceded by nascent transcript cleavage. RNA cleavage appeared to be an obligatory step in the SII activation process. Recombinant SII activated cleavage while a truncated derivative lacking polymerase binding activity did not. Cleavage was not restricted to an elongation complex arrested at this particular site, showing that nascent RNA hydrolysis is a general property of RNA polymerase II elongation complexes. These data support a model whereby SII stimulates elongation via a ribonuclease activity of the elongation complex.

Expression of many genes in eukaryotes requires transcription of extremely large stretches of DNA by RNA polymerase II. During this transcription, RNA polymerase II encounters signals which block full-length primary-transcript synthesis (reviewed by Spencer and Groudine, 1990; Kerppola and Kane, 1991). Transcription *in vitro* using partially purified or defined components has resulted in the identification and characterization of RNA polymerase II transcription arrest sites (Spencer and Groudine, 1990). In some cases, arrest occurs at a bend in the helical axis of template DNA (Kerppola and Kane, 1990). Examples include sites within the first intron of the human *c-myc* gene, the first intron of the human histone H3.3 gene, and an early transcription unit of SV40. It has been suggested that at least part of the information that halts transcription resides in a bend-inducing structural element and that the bent template may provide a steric block to translocation of the enzyme (Kerppola and Kane, 1990).

*In vitro,* the RNA polymerase II elongation complex is extremely stable, as might be expected in order for RNA polymerase II to transcribe very large genes. However, since transcription can become efficiently blocked at numerous sites both *in vivo* and *in vitro,* it is important to understand how an arrested transcription complex can become reactivated for transcription. The transcription of megabase-sized genes and the regulation of growth controlling genes probably relies upon accessory transcription elongation factors to govern the efficiency of primary transcript synthesis.

*This work was supported by a grant (to D. R.) from the University Research Committee of Emory University, American Cancer Society Grant IRG-182, and National Institutes of Health Grant GM-46331.

© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

¶To whom correspondence should be addressed. Tel.: 404-727-3361; Fax: 404-727-2738..

[3]The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Factors that influence transcription elongation by RNA polymerase II have been identified (Sekimizu *et al.,* 1976; Spindler, 1979; Sawadogo *et al.,* 1980; Reinberg and Roeder, 1987; Price *et al.,* 1987; Bengal *et al.,* 1991; Chafin *et al.,* 1991). One such protein is elongation factor SII, which is not required for initiation. SII has been purified from cultured mouse cells (Sekimizu *et al.,* 1976), yeast (Sawadogo *et al.,* 1980), *Drosophila* (Price *et al.,* 1987), HeLa cells (Reinberg and Roeder, 1987; Reines *et al.,* 1989), calf thymus (Rappaport *et al.,* 1987), and bovine brain (Reines, 1991a). cDNAs have been isolated for the mouse (Hirashima *et al.,* 1988), yeast (Hubert *et al.,* 1983; Clark *et al.* 1991), *Drosophila* (Marshall *et al.,* 1990), and human (Yoo *et al.,* 1991) factors.

SII is a 35–38 kDa polypeptide that allows RNA polymerase II to readthrough a pause in the adenovirus major late transcription unit (Reinberg and Roeder, 1987). This pause is important in regulating the viral infectious cycle (Maderious and Chen-Kiang, 1984, Mok *et al.,* 1984). SII also enables RNA polymerase II to readthrough a transcription arrest signal (called site Ia) in the first intron of a human histone gene (Reines *et al.,* 1989; SivaRaman *et al.,* 1990). This site may serve to regulate the gene's expression pattern during the cell cycle.[1] The means by which SII activates transcription elongation is unknown.

Two properties of SII may provide clues to its mechanism of stimulating elongation. SII binds RNA polymerase II (Sawadogo *et al.,* 1980; Horikoshi *et al.,* 1984; Reinberg and Roeder, 1987; Rappaport *et al.,* 1988). SII can also activate a previously unrecognized ribonuclease activity of elongation complex-associated RNA polymerase II (Reines, 1992).[2] In the presence of SII, nascent transcript cleavage shortens the substrate RNA from its 3′-end by a few nucleotides, although more extensive digestion has also been observed.

To determine whether SII's RNA polymerase binding activity and its nuclease activating activity are involved in efficient transcription through intragenic regions, we have examined a discrete population of purified elongation complexes arrested at a well defined site within a human histone gene. We show that transcript cleavage precedes SII-dependent transcript elongation and that an elongation complex containing a shortened RNA is an intermediate in SII activation of RNA chain elongation. A truncated form of SII that is incapable of binding RNA polymerase II cannot activate RNA shortening. These findings support a model in which SII activation of a RNA polymerase 11-associated nuclease is integral to the transcriptional readthroughp rocess. Furthermore, transcript cleavage is not restricted to elongation complexes arrested at this SII-sensitive site in the human histone gene; therefore, nascent RNA cleavage activity is a general property of the RNA polymerase II elongation complex

## MATERIALS AND METHODS

### Proteins and Reagents

RNA polymerase II and SII-free general transcription initiation factors were purified from rat liver as described (Conaway *et al.*, 1987; Reines, 1991a). Yeast inorganic pyrophosphatase was obtained from Sigma. Acetylated bovine serum albumin, T7 RNA polymerase, and *Eco*RI were supplied by Promega Biotec. RNAguard, *Nde*I, *Puu*II, and unlabeled, fast protein liquid chromatography-purified nucleotides were purchased from Pharmacia LKB Biotechnology Inc. [α-$^{32}$P]CTP was purchased from Amersham Corp. Protein concentration was determined with protein assay dye reagent (Bio-Rad) according to the supplier's directions using bovine serum albumin (Sigma, Fraction V) as a standard. DNA templates (pAdTerm-2, Reines *et al.*, 1987; pDNAdML, Conaway and Conaway,

[1] J. Herrmann, L. Zhang, A. Taylor, L. Kedes, and D. Wells, manuscript submitted
[2] M. Izban and D. Luse, manuscript submitted

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

1988) were purified by centrifugation on CsC1-ethidium bromide gradients and linearized by cleavage at theirs ingle *Nde*I site.

### In Vitro Synthesis of SII Protein

An *Eco*RI fragment containing the region encoding mouse SI1 was removed from pSII-3 (Hirashima *et al.*, 1988) and inserted into pGEM2 such that transcription from the T7 promoter yields sense RNA. The resulting plasmid, pGEMSII, was linearized with *Puu*II (for full-length SII) or *Eco*RV (for ΔSII) and transcribed *in vitro* with T7 RNA polymerase. RNA was translated in the presence of [$^{35}$S]$_{L}$-methionine (>l000 Ci/mmol, Du Pont-New England Nuclear) in a wheat germ translation extract (Promega Biotec). Translation reactions were dialyzed into 20 mM HEPES$^{3}$-NaOH, pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol and chromatographed on phosphocellulose as described for bovine brain SII (Reines, 1991a). The amount of *in vitro* translated SI1 protein was estimated 1) by measuring the amount of methionine that was incorporated into a form precipitable in 10% (w/v) trichloroacetic acid, 2) by using 1000 Ci/mmol as the specific activity of labeling methionine, and 3) with knowledge of the cDNA sequence (Hirashima *et al.*, 1988) of SI1 which assigns 12 methionine residues to full-length SII

### RNA Polymerase II Binding Assays

RNA polymerase II (0.5 μg of DEAE-Sephadex A-25 fraction; Reines, 1991a) was mixed with 15 μg of protein A-Sepharose-purified anti-RNA polymerase II IgG (8WG16; Thompson *et al.*, 1989) in binding buffer (22 mM Tris-HC1, pH 7.9,3 mM HEPES-NaOH, pH 7.9, 70 mM KCl, 3% (v/v) glycerol, 0.6 mM EDTA, 2 mM dithiothreitol, 225 μg/ml acetylated-bovine serum albumin). Six μl of fixed Staphylococcus aureus (Bethesda Research Laboratories), washed in binding buffer, were added bring the final volume to 25 μl. Reactions were incubated at 28 °C for 10 min. Precipitates were collected by centrifugation for 2 min in microcentrifuge and were resuspended in 20 μ1 of binding buffer. $^{35}$S-Labeled SII or $^{35}$S-labeled ΔSII were added and incubated for 15 min at 28 °C. Soluble and precipitable proteins were separated by centrifugation for 2 min in a microcentrifuge and were subjected to electrophoresis on 15% polyacrylamide gels (Laemmli, 1970) and fluoro-graphed using sodium salicylate as a fluor (Chamberlain, 1979).

### RNA Polymerase II Elongation Complex Assembly and Isolation

RNA polymerase II elongation complexes arrested at a specific site (Ia) within a human histone gene were assembled from partially purified rat liver RNA polymerase II and general initiation factors as described (Reines *et al.,* 1989). Site Ia is a transcription arrest site that, in the absence of elongation factor SII, halts 40–50% of RNA polymerase II molecules. The template employed was pAdTerm-2 cleaved with *Nde*I. RNA was pulse labeled with 20 μM ATP, 20 μM UTP and ≈0.6 μM [α-$^{32}$P]CTP (>400 Ci/mmol) in the absence of GTP. This results in synthesis of a 14-nucleotide RNA labeled with [$^{32}$P]CMP at positions 2,4,6,9,10, and 12 of the RNA chain. Heparin (10 μg/ml) was added along with unlabeled CTP, GTP, ATP, and UTP. This population of template-engaged nucleoprotein complexes are referred to as elongation complexes. One reaction equivalent is 60 μl. Active elongation complexes were precipitated with an anti-RNA monoclonal antibody (D44, Eilat *et al.*, 1982; Reines, 1991b). Protein A-Sepharose-purified IgG (0.4 μg), and fixed *S. aureus* washed in reaction buffer (20 mM Tris, 3 mM HEPES, pH 7.9, 62 mM KCl, 2.2% polyvinyl alcohol, 3% (v/v) glycerol, 2 mM dithiothreitol, 0.5 mM EDTA, and 0.3 mg/ml acetylated-bovine serum albumin), were added and the complexes were collected by centrifugation in a microcentrifuge for 2 min. These immunoprecipitated elongation complexes were washed by two additional rounds of centrifugation and resuspension in reaction buffer. This preparation is referred to as washed elongation complexes. Various nucleotides, proteins, and salts were added to the washed complexes as indicated. SII was partially purified from

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

bovine brain as described (Reines, 1991a). The purification of rat liver SII employed sequential chromatography on phosphocellulose, AcA 34, hydroxylapatite, and TSK-Φ resins and will be reported elsewhere[4] Reactions were stopped with a SDS-containing buffer (0.2 M Tris-HCl, pH 7.5, 25 mM EDTA, 0.3 M NaC1, 2% (w/v) SDS), and RNA was isolated as described (Reines, 1992).

### Gel Electrophoresis and Autoradiography

RNA was dissolved in 80% (v/v) formamide, 0.025% (w/v) xylene cyanole, and 0.025% (w/v) bromphenol blue in TBE (89 mM Tris, 89 mM boric acid, pH 8.0, 1 mM EDTA), denatured at 95 °C, and subjected to electrophoresis on 30-cm long, 5% polyacrylamide gels in TBE containing 8.3 M urea. Gels were dried and exposed to X-OMAT (Kodak) film at −80 °C with an intensifying screen. $^{32}$P-Labeled reference RNAs were synthesized from pKK34–121 (Thayer and Brosius, 1985) using *Escherichia coli* RNA polymerase (a gift from Dr. M. Chamberlin, University of California, Berkeley).

## RESULTS

### Recombinant SII Synthesized in Vitro Stimulates the Ribo-nuclease Function of an RNA Polymerase II Elongation Complex

To confirm that the addition of SII is sufficient to activate the ribonuclease activity of RNA polymerase II, and to test whether RNA polymerase II-binding by SII is required for this activation, SII and a truncated form of SII (ΔSII) were prepared from a mouse cDNA clone (Hirashima *et al.,* 1988) by *in vitro* transcription and translation in a wheat germ extract. The truncated derivative lacked its carboxyl-terminal 107 amino acids, in which residues important for RNA polymerase II binding reside (Horikoshi *et al.,* 1990; Agarwal *et al.,* 1991). [$^{35}$S]SII and [$^{35}$S]ΔSII were separated from the bulk of the wheat germ translation extract proteins by chromatography on phosphocellulose. These *in vitro* synthesized proteins were analyzed for RNA polymerase II binding activity by testing their ability to coimmunoprecipitate with RNA polymerase II. A monoclonal antibody against the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II (8WG16, Thompson *et al.,* 1989) was used to isolate CTD-containing enzyme from a preparation of partially purified rat liver RNA polymerase II. The immunoprecipitate containing 8WG16 and RNA polymerase II was resuspended with $^{35}$S-labeled SII or ΔSII (Fig. 1A). Efficient precipitation of the full-length protein required both the anti-RNA polymerase II antibody (Fig. 1A, *lanes 3-6*) and RNA polymerase II.[4] ΔSII was not significantly precipitable above background when present at the same concentration at which SII was efficiently precipitated (Fig. 1A, *lanes 7-10*). The relatively high background of precipitable SII was reproducible, detected when protein A-Sepharose was the immunosorbent, and could not be reduced after further washing of the immnoprecipitate.[4] These data independently confirm previous findings in which the region of SII important for RNA polymerase II binding was identified (Horikoshi *et al.,* 1990; Agarwal *et al.,* 1991). Furthermore, binding of 8WG16 to the CTD of RNA polymerase did not prevent the subsequent binding of SII to the enzyme.

Recombinant SII proteins were also tested for their ability to activate SII-dependent nascent transcript cleavage (Fig. 1B). We have recently shown that RNA chains contained in active elongation complexes can be cleaved after exposure to partially purified bovine brain SII (Reines, 1992). Only RNA in an elongation complex and not free RNA was cleaved. The nuclease activity was inhibited by $\alpha$-amanitin (1 $\mu$g/ml). The simplest interpretation of those findings was that RNA polymerase II itself contains this catalytic function.

[4]D. Reines, P. Ghanouni, Q.-Q. Li, and J. Mote, Jr., unpublished results.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Elongation complexes containing [$^{32}$P]RNA were assembled by transcription *in vitro* of a region of the human H3.3 histone gene that arrests transcription with high efficiency (Reines *et al.,* 1987, 1989). Arrested elongation complexes were isolated and washed free of nucleotides by immunoprecipitation using an anti-RNA monoclonal antibody (Reines, 1991a, 1991b). Only full-length recombinant SII (Fig. 1B, *lanes 2 and 3* ) was capable of activating nascent transcript cleavage. Truncated SII (Fig. 1B, *lanes 4 and 5* ) was ineffective in stimulating transcript shortening, even when present at a molar concentration 10-fold higher than that of the full-length protein. Wheat germ translation extracts alone, or extracts programmed with control RNA, were also ineffective in activating the nuclease activity.[4] These data demonstrated that SII was responsible for activating the nuclease activity of the RNA polymerase II elongation complex and that it need not be synthesized in mammalian cells to acquire its function as an activator of RNA cleavage. It also provides evidence that SII's polymerase binding activity is important for the activation of RNA cleavage.

## Transcript Shortening Precedes SII-mediated Elongation Through Site Ia

RNA cleavage by the elongation complex is rapid; all precursor RNA is shortened in less than 1 min at 28 °C (Reines, 1992). In the absence of nucleotides, the half-life of a shortened RNA can be as long as 1 h (Reines, 1992). It was important then, to determine if the nuclease activity was operative under conditions where SII stimulates transcriptional readthrough, *i.e.* in the presence of SII and nucleotides. Since in this experiment we did not wish to deplete the complexes of nucleotides, they were not immunoprecipitated with anti-RNA antibody. A time course of SII-dependent elongation through this region showed that shortened transcripts were detected after 20 s (Fig. 2). The half-life of the smallest detectable intermediate was less than 25 s. Compared to the more extensively cleaved RNAs seen when no nucleotides were present (Reines, 1992 and Fig. 5, *B* and *C*), the nascent RNA in this experiment underwent only limited shortening. Hence, the size and half-life of cleavage products derived from a single RNA differed in the presence and absence of nucleotides (Figs. 2 and 3, this work and Reines, 1992). The brief half-life of cleaved RNAs seen here most likely resulted from their rapid re-extension in the presence of nucleotides, implying that transcript shortening preceded SII activation of elongation. Furthermore, these data prove that transcript cleavage is neither an artifact of nucleotide depletion nor of elongation complex immunoprecipitation.

The accumulation of runoff transcripts after 15 min suggested that the cleaved RNAs served as precursor to full-length RNAs (Fig. 2). To better resolve chain cleavage from chain elongation, we sought an approach that would allow us to more readily detect the products of RNA chain extension through site Ia. Thus, we studied SII-activated readthrough in the presence of a limited set of nucleotides. Immunoprecipitated elongation complexes were washed free of unincorporated nucleotides to reduce the NTP concentrations to less than 50 nM each (Reines, 1992). Washed complexes were then supplied with SII and 800 $\mu$M each of GTP, CTP, and UTP. The 3′ termini of RNAs ending at site Ia map to 4 consecutive T residues on the non-transcribed DNA strand (Reines *et al.,* 1987) as indicated in Fig. *3A.* The template sequence down-stream from site Ia is such that in the presence of SII, UTP, GTP, and CTP transcript Ia (-205 nucleotides) can be extended by only 13-16 nucleotides (Fig. 3A); thus, elongation through site Ia should be readily observed. The kinetics of elongation under these conditions clearly demonstrated that nascent RNA was first cleaved and only subsequently elongated past site Ia (Fig. 3*B*). The prolonged half-life of these cleaved RNAs was probably due to a requirement for the insertion of an A residue into the shortened RNA between the cleavage product terminus (see legend to Fig. 3A) and site Ia (Fig. 3A). Apparently, enough residual ATP was present in the reaction to support this insertion. Alternatively, this elongation could represent the incorporation of a non-cognate

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

nucleotide into RNA. In any case, the simplest interpretation is that transcript cleavage preceded and could be resolved from RNA extension during SII-activated transcript elongation.

These data suggested that RNA chains elongated by RNA polymerase II in an SII-dependent manner must pass through an intermediate that was shorter than the original transcript. This was demonstrated by an experiment using the chain-terminating nucleotide 3′*O*-methyl-GTP. Washed elongation complexes were permitted to elongate their RNA chains in the presence of SII, 3′-*O*-methyl-GTP, UTP, and CTP (Fig. 3*C*, *lane 3*) . If SII allowed the direct extension of transcript Ia without going through a shortened intermediate, transcript Ia would be extended by 12–15 nucleotides before the chain-terminating analog was inserted (Fig. 3*A*). If, however, chain shortening was an obligatory intermediate in the SII stimulation reaction, re-extension of the cleavage product would be prevented since after cleavage the chain-terminating G-residue would be inserted before the transcript could be significantly elongated. This would result in a limited extension product shorter than transcript Ia. Indeed, after 30 min at 28 °C only shortened transcripts were observed (Fig. 3*C*). This was consistent with the idea that incorporation of 3′-*O*-methyl-GMP into the transcript prevented the shortened RNA from serving as a precursor for chain elongation through site Ia. We emphasize that this reaction contained elongation factor SII and all nucleotides necessary for direct extension of transcript *Ia.* Thus, it appears that RNA chain shortening is a prerequisite to transcription through site Ia since preventing the extension of the cleaved RNA did not reveal an alternative means of elongating RNA chains, for example via the *direct* extension of Ia-RNA.

## SII-dependent Chain Shortening Is Not Pyrophosphorolysis

Pyrophosphorolysis, reversal of the nucleic acid biosynthetic reaction, can be carried out by DNA polymerases (Deutscher and Kornberg, 1969; Atkinson *et al.,* 1969) and bacterial and bacteriophage RNA polymerases (Maitra and Hunvitz, 1967; Krakow and Fronk, 1969; Kassavetis *et al.,* 1986; Arndt and Chamberlin, 1990) in the presence of milli-molar levels of pyrophosphate ($PP_i$. Since RNA shortening by RNA polymerase II was dependent upon added SII, it seemed unlikely that cleavage resulted from pyrophosphorolysis (Reines, 1992). Nevertheless, it was important to rule out the possibility that SII acted as a $PP_i$ generator and to show that SII-dependent RNA shortening was distinct from pyrophosphorolysis. After 30 min in the presence of $PP_i$ and MgC12, RNA polymerase II elongation complexes shortened a small but significant fraction of RNA chains (Fig. 4*A*, *lane 3* ) . Since the $^{32}P$ label was present at the 5′-end of these RNA chains, nucleotide residues were removed from their 3′ends. This was an SII-independent, $PP_I$-dependent process that yielded a product distinct from that yielded a product distinctf rom that seen in the SII-dependent, $PP_I$-independent cleavage of nascent RNA chains (Fig. 4*A*, compare *lanes 1* and *3*). The simplest interpretation was that this reaction represented pyrophosphorolysis. Previous work has shown that no significant RNAcl eavage took place when washed complexes were incubated with $MgC1_2$, in the absence of both $PP_i$, and SII (Reines, 1992).

To demonstrate further that we were observing two distinct processes, pyrophosphorolysis and SII-dependent transcript cleavage, we inhibited pyrophosphorolysis by the addition of pyrophosphatase. Pyrophosphatase hydrolyzes inorganic $PP_i$ to orthophosphate and thus, prevents pyrophosphorolysis (Cooperman, 1982; Tabor and Richardson, 1990). Washed elongation complexes were allowed to undergo SII-dependent or $PP_I$-dependent RNA shortening in the presence of pyrophosphatase (Fig. 4*B*). Whereas the extent of $PP_i$-dependent transcript shortening was dramatically reduced in the presence of inorganic pyrophosphatase (Fig. 4*B*, *lanes 3* and *4*, SII-dependent RNA cleavage was unaffected by the enzyme (*lanes I* and *2*). We take this as evidence that washed RNA polymerase II

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

elongation complexes can undergo pyrophosphorolysis and that it is chemically distinct from the more efficient and extensive RNA cleavage activated by SII.

### SII Can Activate the RNA Cleavage Reaction in Other RNA Polymerase II Elongation Complexes

To this point we have observed nascent RNA cleavage in only two elongation complexes, the prominent one arrested at site Ia and a minor, less well-characterized site downstream (+325) in this template (pAdTerm-2, Reines, 1992 and Figs. 2 and 3). We tested the ability of an additional elongation complex to cleave its nascent RNA in response to SII. Washed elongation complexes containing RNA polymerase arrested at site Ia were allowed to extend their transcripts in the presence of added SII, UTP, GTP, and CTP as described above (Fig. 3). The majority of polymerase molecules became arrested at a downstream location where the first A residue was required. Small amounts of readthrough were seen which result in the extension of this RNA by two nucleotides to the next required A residue. These complexes were washed by precipitation and resuspension with anti-RNA antibody and challenged with rat liver SII for varying periods of time (Fig. 5*A*). By 10 min, chain shortening was apparent (Fig. 5*A*, *lane 6*). Thus, an SII-activated RNA-cleavage activity seems to be a general trait of an RNA polymerase II elongation complex. (Rat liver SII was used here since relatively large amounts of highly purified protein can be obtained in a concentrated form during the isolation of rat liver initiation factors. Thus far, purified bovine, human, and rat SII appear functionally indistinguishable.[4])

In some experiments run-off RNAs appeared shortened. This raised the possibility that some RNA polymerase molecules remained in functional elongation complexes for a significant time after they had reached the end of a duplex DNA template. If so, it would provide an opportunity to study RNA cleavage in more elongation complexes arrested at a site distinct from Ia. Like the complexes shown in Fig. 5*A*, these elongation complexes also represent RNA polymerases that were arrested for a reason other than their dependence upon SII for transcription elongation. Labeled runoff RNA was synthesized *in vitro* from two different linear templates (Fig. 5, *B* and *C*) and subjected to immunoprecipitation with anti-RNA monoclonal antibody. The precipitate was washed free of nucleotides and incubated with rat liver SII for varying times. Indeed “runoff” RNAs became progressively shorter with increasing incubation times. A small fraction of the RNAs were shortened by as many as 100 nucleotides judging by their mobility *versus* marker RNAs of known size (Fig. 5*B*). Since the addition of nucleotides resulted in efficient transcript extension (Fig. 5, *B* and *C*, *chase lanes*), cleaved RNA remained in functional elongation complexes. Only a portion of the runoff RNA, which was resolved as a closely spaced doublet (Fig. 5*C*), was capable of being shortened, perhaps indicating that only a fraction of the RNA remained associated with the template and RNA polymerase II in an elongation complex. However, a significant number of RNA polymerase II molecules that synthesized runoff RNA did not appear to dissociate from these DNA termini. Collectively, the data of Fig. *5* show that many different RNA polymerase II elongation complexes possess the ability to cleave their nascent RNA chains in response to elongation factor SII. Therefore, elongation complexes other than those stopped at either SII-dependent transcription arrest sites or sites coincident with a bend in the template can perform this function

## DISCUSSION

We have examined the relationship between SII-activated nascent transcript cleavage and transcription elongation by RNA polymerase II elongation complexes. The most important observation presented here is that nascent RNA cleavage precedes, and appears to be an obligatory intermediate in, SII-mediated transcriptional readthrough of a specific transcription arrest site. The association of nascent RNA cleavage with SII-regulated

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

transcription elongation strongly suggests that the nuclease is causally involved in the readthrough process. The available data, including α-amanitin experiments, suggest that RNA polymerase II harbors the catalytic residues involved in the nuclease activity. This is not surprising since DNA polymerases, reverse transcriptases, RNA polymerase I (Huet *et al.,* 1976), and bacterial RNA polymerase (Surratt *et al.,* 1991) possess intrinsic nuclease activities. It has recently been reported that amino acids in the catalytic center of barnase, a bacterial ribonuclease, are shared with two other bacterial ribonucleases and the second largest sub-units of *Drosophila* and yeast RNA polymerase II (Shirai and Go, 1991). Alternatively, SII may contain all or part of the catalytic site. If so it must interact with RNA polymerase II in a manner that is inhibited, perhaps allosterically, by α-amanitin.

The transcription arrest site studied here lies in a bent region of DNA (Kerppola and Kane, 1990). Evidence suggests that such a DNA helix represents a population in which bent isomers exist in equilibrium with relatively unbent or linear isomers (Koo *et al.,* 1986). A model of SII-assisted readthrough at this site which accommodates this feature of the template has been put forth (Reines, 1992). It states that RNA polymerase II arrested at the site Ia bend prevents isomerization of bent DNA to an elongation-permissive, unbent form. SII, acting as an allosteric ligand, binds the elongation complex and activates a latent nuclease in RNA polymerase II. As a result of shortening the 3′-end of the growing RNA chain, polymerase moves relative to the template allowing renewed interconversion between bent and unbent DNA, followed by the resumption of transcript elongation. The findings presented here fulfill a prediction of this model: namely, that nascent RNA cleavage precedes SII-dependent transcript elongation. Our experiments with the chain terminator, 3′-*O*-methyl-GTP show that even in the presence of SII, and all nucleotides necessary for *direct* transcription from site Ia, elongation does not take place (Fig. 3C). Instead, we observe a new transcription intermediate: an RNA polymerase II elongation complex that bears a cleaved nascent transcript. Although it is possible that RNA cleavage may not accompany readthrough at all SII-dependent arrest sites, it is likely that the short half-life of the cleavage product seen in the presence of nucleotides probably precluded detection of this event in previous studies of SII-mediated readthrough (Reinberg and Roeder, 1987; Rappaport *et al.,* 1987; SivaRaman *et al.,* 1990; Bengal *et al.,* 1991). It will be interesting to learn if other elongation factors also activate nascent transcript cleavage.

Elongation complexes located at template positions other than site Ia were also responsive to SII-activated RNA cleavage. It should be noted that the relative propensity for nascent transcript cleavage differs markedly between complexes. In washed elongation complexes, cleaved RNAs derived from transcript Ia were observed seconds after exposure to SII (Reines, 1992) whereas other complexes cleaved their RNAs slowly requiring many minutes or hours to obtain significant cleavage (Fig. 5 ) . These findings show that a DNA bend is not required for SII activation and suggests that *in vivo* SII could stimulate transcription past other kinds of impediments to elongation. These could include nucleosomes or other DNA-bound proteins. If RNA polymerase “retreats” from a blockage site through RNA shortening, it may be able to sample repeatedly regions of DNA which are only readthrough permissive part of of efficient transcript elongation. This mechanism may explain how the extremely stable RNA polymerase II elongation complex can be assisted in finishing gene transcription through numerous different types of barriers to transcript elongation.

To our knowledge this is the first demonstration of pyrophosphorolysis catalyzed by a specific eukaryotic transcription complex. Furthermore, SII-activated RNA cleavage by the complex is distinct from pyrophosphorolysis in that the former does not require $PP_i$ addition and is insensitive to pyrophosphatase. Thus, SII does not appear to activate the cleavage reaction by serving as a source of $PP_I$ for pyrophosphorolysis. Accordingly, we think that SII-activated RNA cleavage is hydrolytic. The process is initiated presumably when SII

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

associates with the arrested elongation complex. This is consistent with our finding that SII defective in polymerase binding is inactive in nuclease activation.

Recent cloning of a yeast protein (DST$\alpha$) with recA-like, DNA strand transfer activity has shown that it is highly homologous, if not identical, to elongation factor SII (Clark *et al.,* 1991; Kipling and Kearsey, 1991). It was suggested that the strand transfer function of yeast DST$\alpha$/SII may facilitate transcription elongation by promoting displacement of a RNA-DNA hybrid within the transcription bubble and favoring renaturation of the DNA duplex (Kipling and Kearsey, 1991). Perhaps by doing so, SII also potentiates nascent RNA cleavage by making the 3′-end of the RNA accessible nuclease domain of RNA polymerase II where it can be hydrolyzed.

## REFERENCES


Agarwal K, Baek K, Jeon C, Miyamoto K, Ueno A, Yoon H. Biochemistry. 1991; 30:7842–7851. [PubMed: 1868060]

Arndt KM, Chamberlin MJ. J. Mol. Biol. 1990; 213:79–108. [PubMed: 1692594]

Atkinson MR, Deutscher MP, Kornberg A, Russell AF, Moffatt JG. Biochemistry. 1969; 8:4897–4904. [PubMed: 4312461]

Bengal E, Flores O, Krauskopf A, Reinberg D, Aloni Y. Mol. cell. Biol. 1991; 11:1195–1206. [PubMed: 1996086]

Chafin DR, Claussen TJ, Price DH. J. Biol. Chem. 1991; 266:9256–9262. [PubMed: 1851172]

Chamberlain JP. Anal. Biochem. 1979; 98:132–135. [PubMed: 543547]

Clark AB, Dykstra CC, Sugino A. Mol. Cell. Biol. 1991; 11:2576–2582. [PubMed: 1850099]

Conaway JW, Conaway RC. J. Biol. Chem. 1988; 264:2357–2362. [PubMed: 2914911]

Conaway JW, Bond M, Conaway RC. J. Biol. Chem. 1987; 262:8293–8297. [PubMed: 3597375]

Cooperman BS. Methods Enzymol. 1982; 87:526–548. [PubMed: 6129562]

Deutscher MP, Kornberg A. J. Biol. Chem. 1969; 244:3019–3028. [PubMed: 4306290]

Eilat D, Hochberg M, Fischel R, Laskov R. Proc. Natl. Acad. Sci. U. S. A. 1982; 79:3818–3822. [PubMed: 6179087]

Hirashima S, Hirai H, Nakanishi Y, Natori S. J. Biol. Chem. 1988; 263:3858–3863. [PubMed: 3346229]

Horikoshi M, Sekimizu K, Natori S. J. Biol. Chem. 1984; 259:608–611. [PubMed: 6200476]

Horikoshi N, Sekimizu K, Natori S. J. Biol. Chem. 1990; 265:11854–11857. [PubMed: 1973165]

Hubert J-C, Guyonvarch A, Kammerer B, Exinger F, Liljelund P, Lacroute F. EMBO J. 1983; 2:2071–2073. [PubMed: 6139279]

Huet J, Wyers F, Buhler J-M, Sentenac A, Fromageot P. Nature. 1976; 261:431–433. [PubMed: 778628]

Kassavetis GA, Zenter PG, Geiduschek EP. J. Biol. Chem. 1986; 261:14256–14265. [PubMed: 2429954]

Kerppola TK, Kane CM. Biochemistry. 1990; 29:269–278. [PubMed: 2157481]

Kerppola TK, Kane CM. FASEB J. 1991; 5:2833–2842. [PubMed: 1916107]

Kipling D, Kearsey SE. Nature. 1991; 353:509. [PubMed: 1922360]

Koo H-S, Wu H-M, Crothers DM. Nature. 1986; 320:501–506. [PubMed: 3960133]

Krakow JS, Fronk E. J. Biol. Chem. 1969; 244:5988–5993. [PubMed: 4310829]

Laemmli UK. Nature. 1970; 227:680–685. [PubMed: 5432063]

Maderious A, Chen-Kiang S. Proc. Nat. Acad. Sci. U. S. A. 1984; 81:5931–5935.

Maitra U, Hurvitz J. J. Biol. Chem. 1967; 242:4897–4907. [PubMed: 4293779]

Marshall TK, Guo H, Price DH. Nucleic. Acids Res. 1990; 18:6293–6298. [PubMed: 2243775]

Mok M, Maderious A, Chen-Kiang S. Mol. Cell. Biol. 1984; 4:2031–2040. [PubMed: 6209554]

Price DH, Sluder AE, Greenleaf AL. J. Biol. Chem. 1987; 262:3244–3255. [PubMed: 3818640]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Price DH, Sluder AE, Greenleaf AL. Mol. Cell. Biol. 1989; 9:1465–1475. [PubMed: 2725511]

Rappaport J, Reinberg D, Zandomeni R, Weinmann R. J. Biol. Chem. 1987; 262:5227–5232. [PubMed: 3558392]

Rappaport J, Cho K, Saltzman A, Prenger J, Golomb M, Weinmann R. Mol. Cell. Biol. 1988; 8:3136–3142. [PubMed: 3145407]

Reinberg D, Roeder RG. J. Biol. Chem. 1987; 262:3331–3337. [PubMed: 3818644]

Reines D. J. Biol. Chem. 1991a; 266:10510–10517. [PubMed: 1709935]

Reines D. Anal. Biochem. 1991b; 196:367–372. [PubMed: 1723251]

Reines D. J. Biol. Chem. 1992; 267:3795–3800. [PubMed: 1371280]

Reines D, Wells D, Chamberlin MJ, Kane CM. J. Mol. Biol. 1987; 196:299–312. [PubMed: 3656448]

Reines D, Chamberlin MJ, Kane CM. J. Biol. Chem. 1989; 264:10799–10809. [PubMed: 2471707]

Sawadogo M, Sentenac A, Fromageot P. J. Biol. Chem. 1980; 255:12–15. [PubMed: 6985606]

Sekimizu K, Kobayashi N, Mizuno D, Natori S. Biochemistry. 1976; 15:5064–5070. [PubMed: 990265]

Shirai T, Go M. Proc. Natl. Acad. Sci. U. S . A. 1991; 88:9056–9060. [PubMed: 1924368]

SivaRaman L, Reines D, Kane CM. J. Biol. Chem. 1990; 265:14554–14560. [PubMed: 2387869]

Spencer C, Groudine M. Oncogene. 1990; 5:777–785. [PubMed: 2193290]

Spindler SR. Biochemistry. 1979; 18:4042–4048. [PubMed: 486411]

Surratt CK, Milan SC, Chamberlin MJ. Proc. Natl. Acad. Sci. U. S. A. 1991; 88:7983–7987. [PubMed: 1716768]

Tabor S, Richardson CC. J. Biol. Chem. 1990; 265:8322–8328. [PubMed: 2159476]

Thayer GC, Brosius J. Mol. Gen. Genet. 1985; 199:55–58. [PubMed: 3889550]

Thompson NE, Steinberg TH, Aronson DB, Burgess RR. J. Biol. Chem. 1989; 264:11511–11520. [PubMed: 2472398]

Yoo O, Yoon H, Baek K, Jeon C, Miyamoto K, Ueno A, Agarwal K. Nucleic Acids Res. 1991; 19:1073–1079. [PubMed: 1708494]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

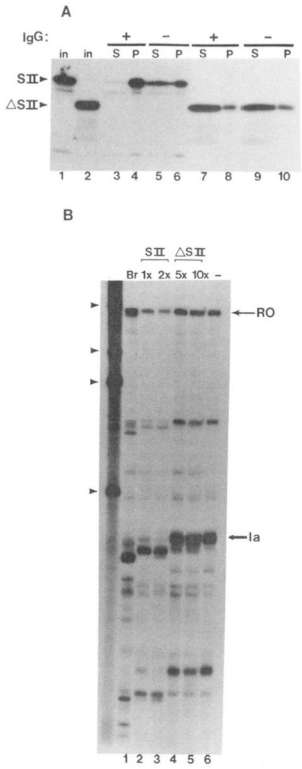


**FIG. 1. Activity of *in vitro* synthesized, recombinant mouse SII and truncated SII (ΔSII)**

*Panel A,* RNA polymerase II binding assay. $^{35}$S-Labeled, phosphocellulose-purified SII and ΔSII were resuspended at a concentration of 4 pM with 8WG16-immunoprecipitated rat liver RNA polymerase II at 28 °C for 15 min. Precipitable (*P*) and soluble ( *S* ) fractions were separated and run on a polyacrylamide gel. In control samples (*lanes 5, 6, 9*, and *10*) anti-RNA polymerase II IgG was omitted. The amount of translation product put into each reaction (*in*) is displayed in *lanes 1* and *2* for SII and ΔSII, respectively. *Panel B*, nascent transcript cleavage assay. Two μg of partially purified bovine brain SII (*Br*, *lane 1* ) , *in vitro* synthesized mouse SII (*lanes 2* and *3*), *in vitro* synthesized mouse ΔSII (*lanes 4* and *5*), or buffer (−, *lane 6* ) were mixed with washed elongation complexes and $MgC1_2$ and incubated for 30 min at 28 °C. The amount of *in vitro* synthesized SII or ΔSII added to each reaction is indicated by *1*× , *2*× , *5*× , and *10*× where × is approximately 50 amol. In the absence of nucleotides, the size of a cleaved nascent RNA is inversely proportional to the concentration of SII.[4] Hence, shorter product was obtained in the presence of bovine brain (*lane 1*) compared to that seen with recombinant mouse SII (*lanes 2* and *3*) since more SII activity was present in the former than the latter. Runoff RNA (*RO*) and RNA resulting from transcription arrest at sire Ia *(Ia)* are indicated. Marker RNAs of 540, 420, 380 and nucleotides (*top* to *bottom*) are indicated with *arrowheads.*

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

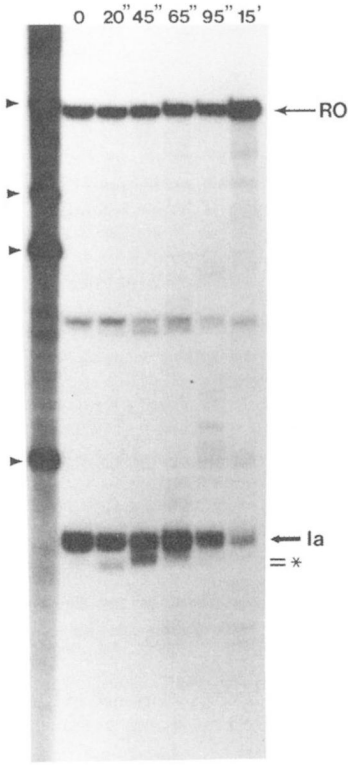


**FIG. 2. Time course of transcription through site Ia in the presence of SII and all four nucleotides**

Six reaction equivalents of washed elongation complexes were incubated with bovine brain SII (12 $\mu$g) and 800 $\mu$M each of ATP, GTP, CTP, and UTP. After 20, 45,65, 95, and 900 s at 28 °C, one reaction equivalent was removed, and RNA was isolated for electrophoresis. Two shortened intermediates are indicated by an *asterisk*. The identity of the other RNAs are as indicated in the legend to Fig. 1*B*.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

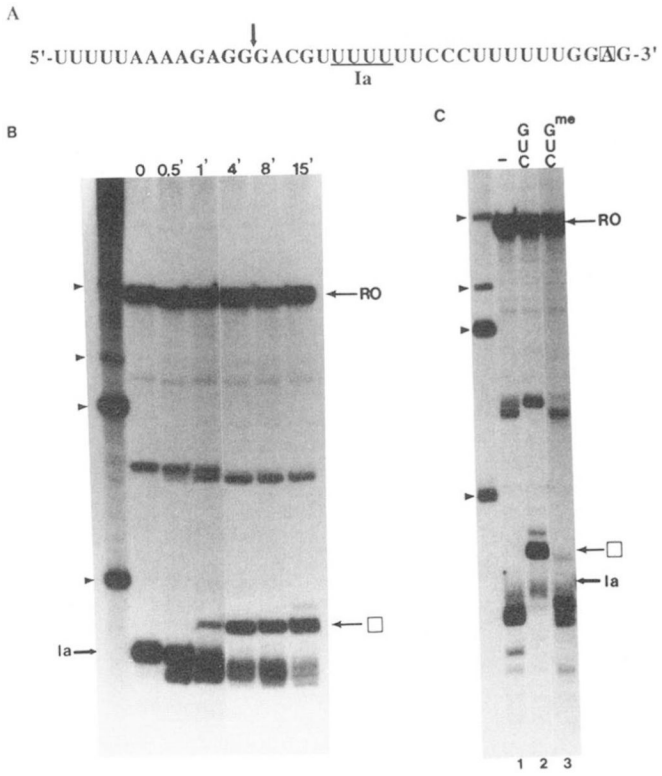


**FIG. 3. Transcript shortening precedes SII-mediated transcription elongation**

*Panel A*, RNA sequence around site Ia within the first intron of the human histone gene. The position of the 3′-ends formed when RNA polymerase II stops transcription are *underlined* and are collectively referred to as site Ia (Reines *et al.,* 1987). The first A residue downstream of site Ia is *boxed (□).* The tentative site of cleavage which generates the major product (Reines, 1992) seen when washed elongation complexes were treated with SII (Fig. 3*C*, *lane 1* ) is indicated by an *arrow*.[4] *Panel B*, transcription through site Ia after the addition of SII, GTP, UTP, and CTP. Washed elongation complexes were incubated with bovine brain SII and 800$\mu$M each of UTP, CTP, and GTP. After 0, 0.5, 1,4,8, and 15 min at 28 °C, aliquots were withdrawn and labeled RNA was isolated for electrophoresis. The RNA that extends to the first A residue down-stream from site Ia is indicated *(□).* The migration position of other RNAs are as indicated in the legend to Fig. 1*B*. *Panel C*, transcription through site Ia in the presence of 3′-*O*-methyl-GTP. Washed elongation complexes were incubated with 800 $\mu$M each of UTP and CTP, bovine brain SII, and either 800 $\mu$M GTP (*lane 2*) or 830 $\mu$M 3′-*O*-methyl-GTP (*lane 3*), for 30 min at 28 °C. RNA was isolated and analyzed by electrophoresis and autoradiography. The migration position of transcript Ia, run in an adjacent lane, is shown *(Ia).* Other RNAs are identified as described in the legend to Fig. 3*B*.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

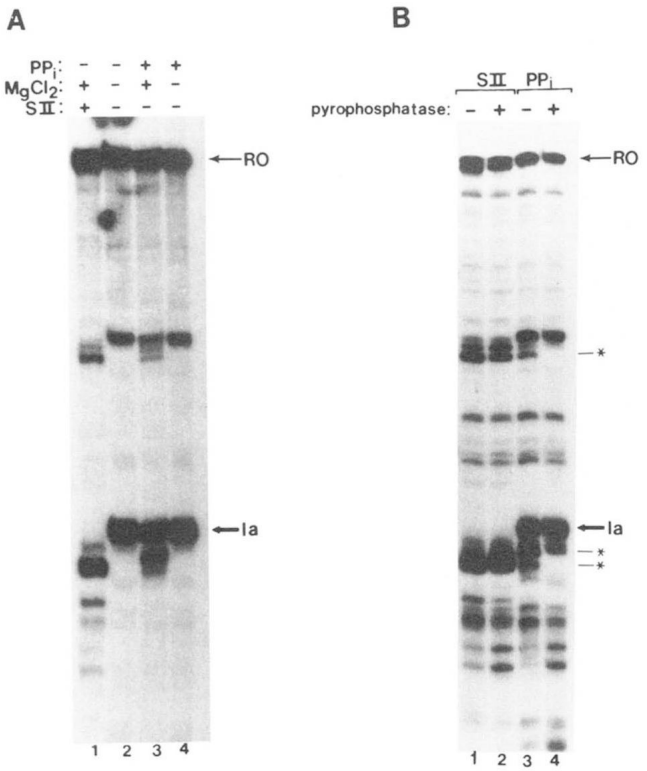


**FIG. 4. RNA polymerase II elongation complexes can carry out pyrophosphorolysis**

*Panel A*, pyrophosphate-dependent transcript shortening. Washed elongation complexes were incubated for 30 min at 28 °C with 7 mM $MgCl_2$, and bovine brain SII (2 $\mu$g), or 1.8 mM sodium pyrophosphate as indicated above the figure. RNA was isolated and analyzed by electrophoresis and autoradiography. *Panel B*, effect of pyrophosphatase on transcript cleavage. Washed elongation complexes were mixed with 7 mM $MgC1_2$ and either bovine brain SII (2 $\mu$g, *lanes 1* and *2*) or 1.5 mM sodium pyrophosphate (*lanes 3* and *4* ) in the presence (+) or absence (−) of 10 units of yeast inorganic pyrophosphatase. (One unit of inorganic pyrophosphatase liberates 1.0 $\mu$mol of inorganic orthophosphate/min.) The reactions were then incubated at 28 °C for 30 min before RNA was isolated for electrophoresis. Three pyrophosphatase-sensitive RNAs are indicated by *asterisks.*

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



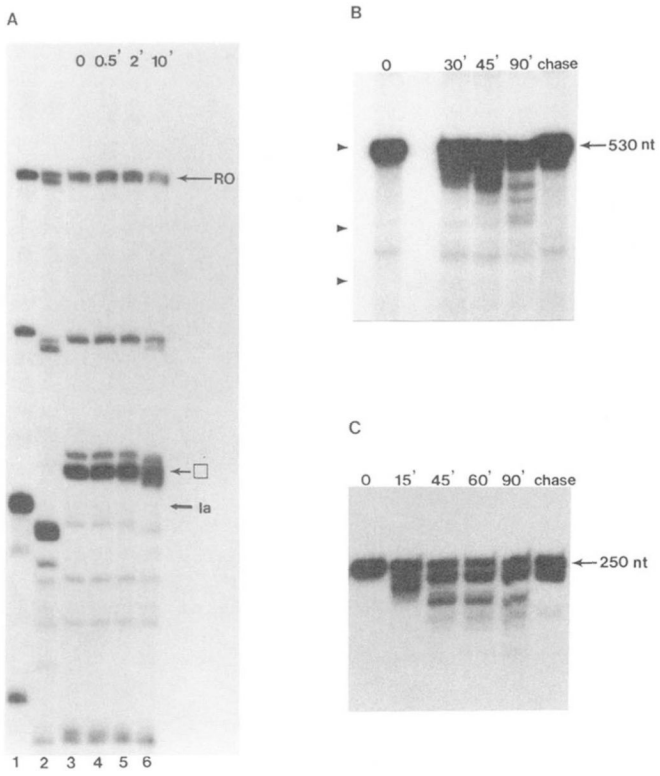


**FIG. 5. Other elongation complexes possess ,SII-activated nascent transcript cleavage activities**

*Panel A*, an RNA polymerase II elongation complex arrested downstream of site Ia canc leave its nascent RNA. Elongation complexes arrested at site Ia were assembled and washed (*lane 1* ) as described under "Materials and Methods." Washed complexes were incubated at 28 °C with SII (*lane 2) or* SII and UTP, CTP, and GTP for 20 min (*lanes 3–6*) as described in the legend to Fig. 3*B*. These "ATP-starved" complexes (*lanes 3–6*) were washed by immunoprecipitation and incubated for 0 (*lane 3*), 0.5 (*lane 4*), 2 (*lane5*), or 10 min (*lane 6*) at 28 °C with approximately $5 \times 10^{-4}$ absorbance units (280 nm) of rat liver SII (TSK-phenyl 5-PW fraction). Samples were prepared for electrophoresis and autoradiography as described under "Materials and Methods." **Panels B** and *C*, runoff transcript cleavage. The plasmids pAdTerm-2 (*B*) or pDNAdML (*C*) were linearized with *Nde*I and transcribed *in vitro* with rat liver RNA polymerase II and general initiation factors. Reactions containing runoff RNAs (*530 nt* and *250 nt*) pulse-labeled with [α-$^{32}$P]CMP were subjected to immunoprecipitation with anti-RNA monoclonal antibody. Precipitates were washed and incubated with approximately $5 \times 10^{-4}$ absorbance units (280 nm) of rat liver SII (TSK-phenyl 5-PW fraction) and incubated at 28 °C. After the indicated number of minutes, aliquotsw ere withdrawn and RNAw as isolated for electrophoresis. An additional sample (*chase*) from each reaction was withdrawn (at 45 min, *panel B*, or at 60 min, *panel C*) and adjusted to 800 $\mu$M in all four nucleotides, incubated for an additional 15 miant 28 °C ,and RNwAa s prepared for electrophoresis. The migration position of marker RNAs of 540, 420, and 380 nucleotides (from top to bottom) are indicated by *arrowheads* in *panel B*. *nt*. nucleotides.