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Elongation factor SII-dependent transcription by RNA polymerase II through a sequence-specific DNA-binding protein

(*lac* repressor/RNA cleavage)

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ABSTRACT **In eukaryotes the genetic material is contained within a coiled, protein-coated structure known as chromatin. RNA polymerases must recognize specific nucleoprotein assemblies and maintain contact with the underlying DNA duplex for many thousands of base pairs. Template-bound *lac* operon repressor from *Escherichia coli* arrests RNA polymerase II *in vitro* and *in vivo* [Kuhn, A., Bartsch, I. & Grummt, I. (1990) *Nature (London)* 344, 559–562; Deuschele, U., Hipskind, R. A. & Bujard, H. (1990) *Science* 248, 480–483]. We show that in a reconstituted transcription system, elongation factor SII enables RNA polymerase II to proceed through this blockage at high efficiency. *lac* repressor-arrested elongation complexes display an SII-activated transcript cleavage reaction, an activity associated with transcriptional readthrough of a previously characterized region of bent DNA. This demonstrates factor-dependent transcription by RNA polymerase II through a sequence-specific DNA-binding protein. Nascent transcript cleavage may be a general mechanism by which RNA polymerase II can bypass many transcriptional impediments.**

Eukaryotic RNA polymerases must access and transcribe the genetic information contained within chromatin. Thus, these enzymes must transcribe DNA enveloped in histone and nonhistone proteins. Much work has focused on transcription through nucleosome-packaged templates (reviewed in ref. 1). RNA polymerases, including RNA polymerase II, readily transcribe through some nucleosomes (2–5). In some cases, however, nucleosomes impede RNA elongation (6), implying a role for accessory elongation factors. In fact, transcription through nucleosomes can be enhanced slightly by elongation factor SII (7). Less is known about *sequence-specific* DNA-binding proteins that block RNA elongation. TTFI is a DNA-binding protein that terminates transcription by RNA polymerase I but does not stop RNA polymerases II or III, *Escherichia coli* RNA polymerase, or phage RNA polymerase (8). Thus, different elongation complexes respond differently to a DNA-bound protein. Conversely, one RNA polymerase may be able to read through some but not all DNA-bound proteins. For example, the *E. coli lac* operon repressor arrests RNA polymerase II but TTFI does not (8, 9).

Some genes transcribed by RNA polymerase II contain internal enhancer elements that serve as protein binding sites (10–12). If these proteins block transcription by RNA polymerase, cellular factors may be required to reactivate the arrested transcription complex. Control of gene expression at the level of transcription elongation has been shown for many class II genes (reviewed in ref. 13). The role of DNA-binding proteins in these regulatory mechanisms is unknown.

lac repressor is a well-studied sequence-specific DNA-binding protein. Transcript elongation by *E. coli* RNA polymerase and RNA polymerase II is blocked by DNA-bound repressor (8, 9, 14, 15). DNA-bound *Eco*RI restriction endonuclease can also block transcription by *E. coli* RNA polymerase (16). Here we have used *lac* repressor as a model to study transcription on simple nucleoprotein templates.

SII is perhaps the best studied eukaryotic transcription elongation factor (reviewed in refs. 17 and 18). It binds RNA polymerase II (19–22) and permits transcription past arrest signals *in vitro* (21, 23–27). It activates a latent ribonuclease activity in template-engaged elongation complexes (28, 29). This RNA cleavage was shown to be associated with SII-mediated readthrough of an elongation block in a human gene (30). SII may operate by activating transcript cleavage upstream of a transcriptional block, thereby allowing RNA polymerase to renew elongation downstream (28–30). Since numerous RNA polymerase II elongation complexes display SII-activated nascent transcript cleavage (28–30), it is plausible that SII facilitates elongation through other types of transcriptional blockages. We have tested this idea by asking whether SII can facilitate transcription through a DNA-bound protein and if so, whether RNA cleavage is involved in the process. We found that repressor-arrested RNA polymerase II is stable and can be activated by SII for transcription through *lac* repressor. It displays the SII-stimulated nuclease demonstrated for elongation complexes arrested by other means (28–30). Hence, DNA-bound protein represents another kind of transcriptional block at which SII can activate the nuclease function of an elongation complex and potentiate readthrough.

MATERIALS AND METHODS

RNA polymerase II and initiation factors were purified from rat liver (31) except for α, which was expressed in *E. coli* (32). SII was synthesized from a mouse cDNA (33) by *in vitro* transcription and translation and purified from the wheat germ translation extract by phosphocellulose chromatography (30). Homogeneous calf thymus SII (23) was provided by R. Weinmann (Wistar Institute, Philadelphia). SII was partially purified from bovine brain (34) and rat liver (30). RNAguard, ultrapure NTPs, and 3′-*O*-methyl-GTP were from Pharmacia LKB Biotechnology. [α-^{32}P]CTP was from Amersham. pAdLac was made by inserting an *Eco*RI–*Hin*dIII fragment from pDNAdML (35) into *Hin*dIII- and *Eco*RI-cut pUC19, which contains the *lac* operator (36).

Transcription was initiated with 50 ng of *Pvu* II-cut pAdLac, initiation factors, and RNA polymerase II in the presence of ATP, UTP, and [α-^{32}P]CTP (24), yielding a 14-nucleotide RNA in initiated transcription complexes. Unless otherwise indicated, each 60-μl reaction mixture was made 50 nM in repressor monomer (Stratagene; 0.1 μg per reaction)

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Abbreviation: IPTG, isopropyl β-D-thiogalactoside.

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and incubated at 28°C for 15 min. Heparin (10 μg/ml) and CTP, GTP, ATP, and UTP (800 μM each) were added and incubation was continued for 10 min at 28°C to generate repressor-arrested complexes. These complexes were precipitated by adding 1.2 μg of anti-RNA monoclonal IgG (34, 37) and incubating for 15 min at 4°C. Ten microliters of fixed *Staphylococcus aureus* (BRL/Life Technologies, Gaithersburg, MD) washed in reaction buffer [20 mM Tris/3 mM Hepes, pH 7.9/62 mM KCl/2.2% (vol/vol) poly(vinyl alcohol)/3% (vol/vol) glycerol/2 mM dithiothreitol/0.5 mM EDTA/acetylated bovine serum albumin at 0.3 mg/ml] was added, and the mixture was incubated for 15 min at 4°C. Complexes were collected by centrifugation in a microcentrifuge for 2 min and washed by two rounds of centrifugation and resuspension in an equivalent volume of reaction buffer. We refer to this material as washed elongation complexes. Reactions were stopped with SDS. RNA was isolated and electrophoresed on 7% (19:1 acrylamide/bisacrylamide) gels.

For RNA sequencing, ^{32}P-labeled runoff RNA was synthesized from *Pvu* II-cut pAdLac, isolated, and cleaved with base-specific ribonucleases (38) and alkali obtained from United States Biochemical.

A 280-bp *Pvu* II–*Hin*dIII fragment containing the *lac* repressor-binding site from pAdLac was labeled with [γ-^{32}P]-ATP and polynucleotide kinase. DNA (50 ng) was incubated with protein in 60 μl of reaction buffer containing heparin (10 μg/ml) at 28°C for 30 min. Identical results were obtained when $MgCl_2$ (7 mM) was also included in the binding reactions (unpublished data). Ten microliters of each reaction mixture was applied to a 5% (37:1 acrylamide/bisacrylamide) gel in 10 mM Tris·HCl, pH 8.0/1 mM EDTA.

RESULTS

***lac* Repressor Blocks Transcription by RNA Polymerase II.** The *E. coli lac* repressor can impede transcription by RNA polymerase II (8, 9). To study *lac* repressor-arrested elongation complexes, we constructed a template containing the adenovirus major late promoter 70 bp upstream from a repressor binding site (Fig. 1). Transcription by RNA polymerase II employed partially purified rat liver initiation factors (31). This fractionated system enables the study of elongation factor-dependent transcription *in vitro* (24). RNA was pulse-labeled and synthesis was limited to a single round. *lac* repressor was incubated with complexes bearing a 14-nucleotide RNA and chains were then extended. A 65-nucleotide arrested RNA was synthesized in the presence of *lac* repressor (Fig. 2*A*). This confirmed the findings of others (8, 9) and was consistent with the idea that elongation by RNA polymerase II was arrested at the occupied *lac* repressor binding site. Approximately 50% of the RNA polymerases stopped chain elongation at this site in the presence of 5 nM repressor (Fig. 2*A*, lane 4). Complete arrest was not observed even in the presence of 1 μM repressor (Fig. 2*A*, lane 1).

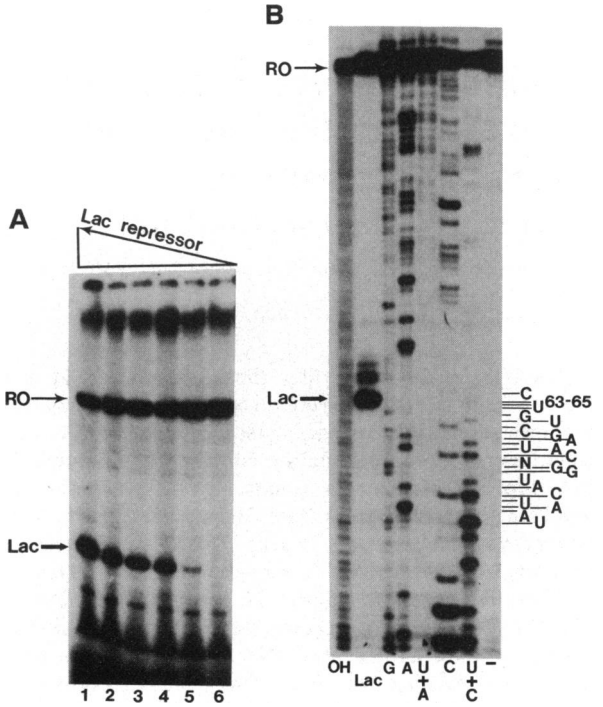

FIG. 2. (*A*) Arrest of transcription by *lac* repressor. *lac* repressor (1 μM, lane 1; 500 nM, lane 2; 50 nM, lane 3; 5 nM, lane 4; 0.5 nM, lane 5) or buffer (lane 6) was incubated with initiated complexes assembled on pAdLac, and RNAs were extended in the presence of heparin and NTPs. Runoff (RO) and arrested RNA (Lac) are indicated. (*B*) Mapping of the 3′ end of arrested RNA. Labeled runoff RNA synthesized from *Pvu* II-cut pAdLac was purified (−) and cleaved with alkali (OH) or RNases T1 (lane G), U2 (lane A), PhyM (lane U+A), CL_3 (lane C), or *Bacillus cereus* (lane U+C). Lane designations indicate base cleavage preferences. Specificities for some nucleases are not absolute—e.g., cleavage at U-54 (N) was not observed. Arrested RNA (Lac) was analyzed with the digestion products.

Using base-specific RNases (38), we mapped the 3′ ends of the arrested RNA. The majority of RNAs terminated at the U residues at positions +63, 64, and 65 (Figs. 1 and 2*B*). This placed the 3′ ends of arrested RNAs ≈10 bp on the nontemplate strand and ≈4 bp on the template strand from the *lac* repressor DNase I footprint (39). Thus, it is likely that RNA polymerase II and *lac* repressor make contact in the arrested complex.

SII Enables Transcription Through the *lac* Repressor Binding Site. The addition of SII to repressor-arrested elongation complexes reduced the half-life of arrested RNA to <15 min (Fig. 3*A*). "Chasing" of arrested RNAs into full-length

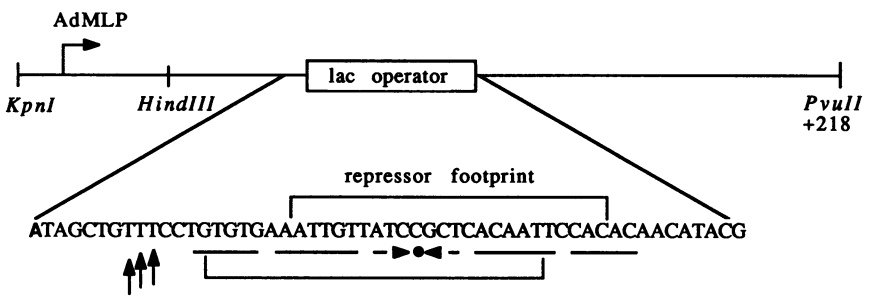

FIG. 1. Transcription template. The bent arrow indicates the transcription initiation site of the adenovirus major late promoter. Operator DNA is indicated by broken arrows. The upward-pointing arrows show the 3′ ends of the major repressor-arrested RNAs. The DNase I footprint of *lac* repressor (39) is indicated by brackets.

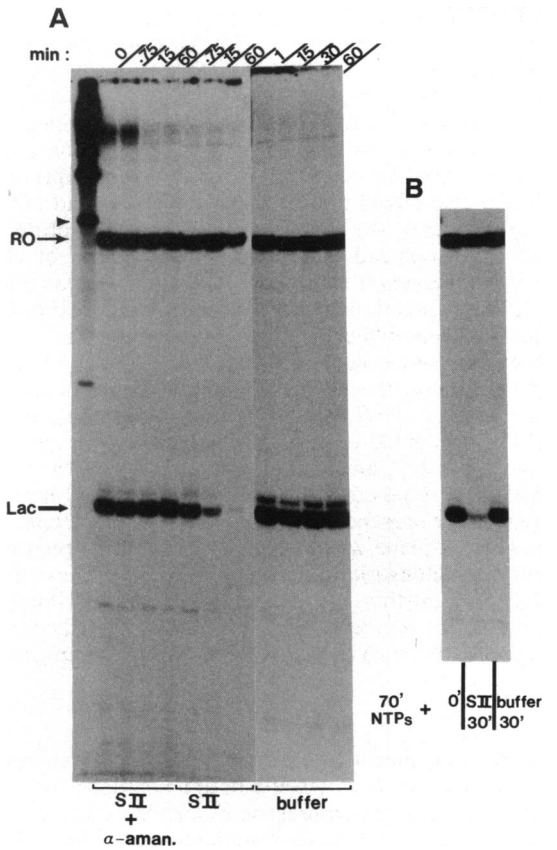

FIG. 3. (*A*) Elongation factor SII enables transcription through *lac* repressor. Arrested complexes were incubated with rat liver SII (phenyl fraction, ≈100 ng, assuming $A_{280}^{1\%}$ of 10.0) or buffer for the indicated times (min). One reaction mixture received α-amanitin (1 μg/ml). The arrowhead indicates a 260-nucleotide marker RNA. (*B*) Stability of repressor-arrested RNA polymerase II elongation complex. Arrested complexes were assembled and incubated with all four NTPs for 70 min. Reactions were stopped (0'), or continued for 30 min with SII or buffer.

transcripts was virtually quantitative (Fig. 3*A*, SII, 60 min) and amanitin-sensitive (Fig. 3A, SII + α-amanitin). Thus, SII enabled RNA polymerase II to overcome an obstacle to transcription created by a DNA-binding protein. To test whether repressor-arrested elongation complexes disassembled after prolonged incubation in the absence of SII, samples were incubated at 28°C for an additional hour after the synthesis of arrested RNA. SII was then added to test the ability of arrested-RNA chains to be elongated. These RNAs were efficiently extended after SII challenge (Fig. 3*B*, SII 30'). Therefore, repressor-arrested complexes remained intact and potentially active for at least 1 hr under these conditions.

The inducer isopropyl β-D-thiogalactoside (IPTG) increases the rate of dissociation of repressor from DNA (40). The half-life of arrested RNA was reduced to <5 min when IPTG was added to arrested complexes (Fig. 4*A*), demonstrating that the arrested complex does not require SII to be reactivated once the blockage has been removed.

We tested whether SII, like IPTG, activated transcription because it antagonized the repressor–DNA interaction. Conceivably, SII could directly remove repressor from DNA. Repressor was mixed with DNA in the presence and absence of SII. Repressor-bound and unbound DNA were separated by electrophoresis on a nondenaturing polyacrylamide gel (41). SII had no observable effect on the apparent affinity of *lac* repressor for DNA (Fig. 4*B*).

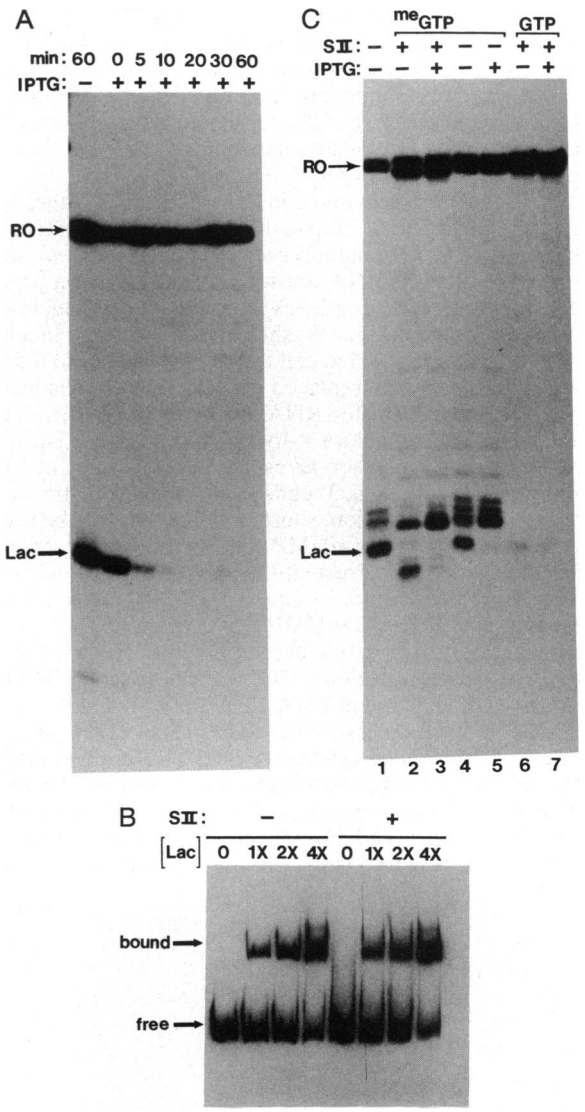

FIG. 4. (*A*) IPTG releases repressor-arrested RNA polymerase II. Repressor-arrested complexes were synthesized, IPTG (3 mM) was added, and samples were removed at the indicated times. A control was incubated for 60 min in the absence of IPTG. (*B*) SII does not affect DNA binding by repressor. Reaction mixtures with variable concentrations of repressor (X = 2 nM) received buffer or rat liver SII (2 μg of phosphocellulose fraction). Free and bound DNA were separated on a nondenaturing gel. (*C*) SII challenge of IPTG-treated complexes. Repressor-arrested complexes were prepared and washed in the presence of 50 nM *lac* repressor. Washed complexes (lane 1) were incubated with 4 mM IPTG (lanes 3, 5, and 7) or H_2O (lanes 2, 4, and 6) for 10 min. Reaction mixtures were made 7 mM in $MgCl_2$, 770 μM each in ATP, UTP, and CTP, and either 800 μM in 3'-*O*-methyl-GTP (lanes 2–5) or 770 μM in GTP (lanes 6 and 7). Reaction mixtures received rat liver SII (2 μg of phosphocellulose fraction; lanes 2, 3, 6, and 7) or buffer (lanes 4 and 5) and were incubated for 10 min.

Repressor-Arrested Complexes Carry out SII-Dependent Nascent Transcript Cleavage. RNA polymerase II elongation complexes cleave nascent transcripts at a number of locations (28–30). This nuclease function is readily detected in the absence of NTP substrate and is activated by elongation factor SII. To test whether elongation complexes arrested at the *lac* repressor also undergo this reaction, we purified them by using an anti-RNA monoclonal antibody (34, 37). Immunoprecipitated complexes can be depleted of nucleotides by repeated washings. RNA cleavage by arrested complexes was observed upon addition of mouse SII synthesized *in vitro*

(Fig. 5A, rMus), partially purified rat liver SII (Fig. 5A, RL), partially purified bovine brain SII (Fig. 5A, BB) or calf thymus SII purified to apparent homogeneity (Fig. 5 B and C). Cleavage of a significant fraction of the runoff transcript was also apparent (Fig. 5B), consistent with previous work showing that active elongation complexes reside at the end of linear DNA duplexes (30).

RNA cleavage is involved in SII-mediated readthrough of an arrest signal in a human histone gene (30). To see if cleavage also accompanies repressor readthrough, we sought to trap the cleaved RNA during the SII-activation process. Repressor-arrested complexes were depleted of nucleotides by immunoprecipitation. Washed complexes were incubated in the presence of purified calf thymus SII and all four NTPs, except that UTP was replaced by 3′-dUTP. This nucleotide serves as a substrate for RNA polymerases and is a chain-terminator, since it lacks a 3′-hydroxyl group (42). The RNAs resulting from repressor arrest are a collection of chains having slightly differing 3′ ends (Figs. 1 and 2B). If cleavage preceded chain extension, shortened RNAs would be trapped by incorporation of 3′-dUMP and would not be extended through the original arrest site. If, however, polymerization proceeds in the absence of cleavage, arrested RNAs should be lengthened before 3′-dUMP is incorporated (Fig. 1). This approach has been used before to trap intermediates in SII-activated transcription (30). In the absence of NTPs, extensive SII-dependent RNA cleavage was again seen (Fig. 5B, lane 5). In the presence of ATP, CTP, GTP, and UTP, elongation proceeded efficiently, yielding runoff transcripts (Fig. 5B, lane 3), demonstrating that arrested complexes were stable to immunoprecipitation. When UTP was replaced with 3′-dUTP, we observed SII-dependent RNA shortening (Fig. 5B, lane 4). Compared with controls (Fig. 5B, lanes 1 and 2), almost no arrested RNAs became lengthened under these conditions. A time course of RNA shortening (Fig. 5C) showed that the cleaved intermediate was produced early in the SII-activation process.

To determine if a transcription complex arrested at this template location *must* cleave its transcript upon reactivation, we stripped *lac* repressor from washed complexes with IPTG and challenged the complexes with SII and NTPs. In this experiment, the chain-terminating nucleotide 3′-*O*-methyl-GTP replaced GTP to permit detection of cleaved RNA intermediates. Recall that after repressor was removed with IPTG, elongation became SII independent (Fig. 4A). In complexes from which repressor was removed, arrested RNA was extended in the absence of SII until 3′-*O*-methyl-GMP was incorporated (Fig. 4C, compare lane 4 with lane 5). In the presence of SII, complexes that were not treated with IPTG and contained repressor cleaved their arrested RNAs (Fig. 4C, lane 2). Conversely, templates from which repressor had been removed with IPTG demonstrated little RNA cleavage in the presence of SII (Fig. 4C, lane 3). Hence, RNA cleavage took place when SII was required for readthrough but not when elongation was SII independent. These findings strongly suggest that RNA cleavage is causally involved in readthrough of *lac* repressor, as seen previously for readthrough of a transcription-arrest signal in a human gene (30).

DISCUSSION

We have confirmed that RNA polymerase II transcription can be arrested *in vitro* by DNA-bound *lac* repressor. A prior report suggested that repressor blockade resulted in transcription termination or its functional equivalent (9). This conclusion was drawn in part from the fact that arrested

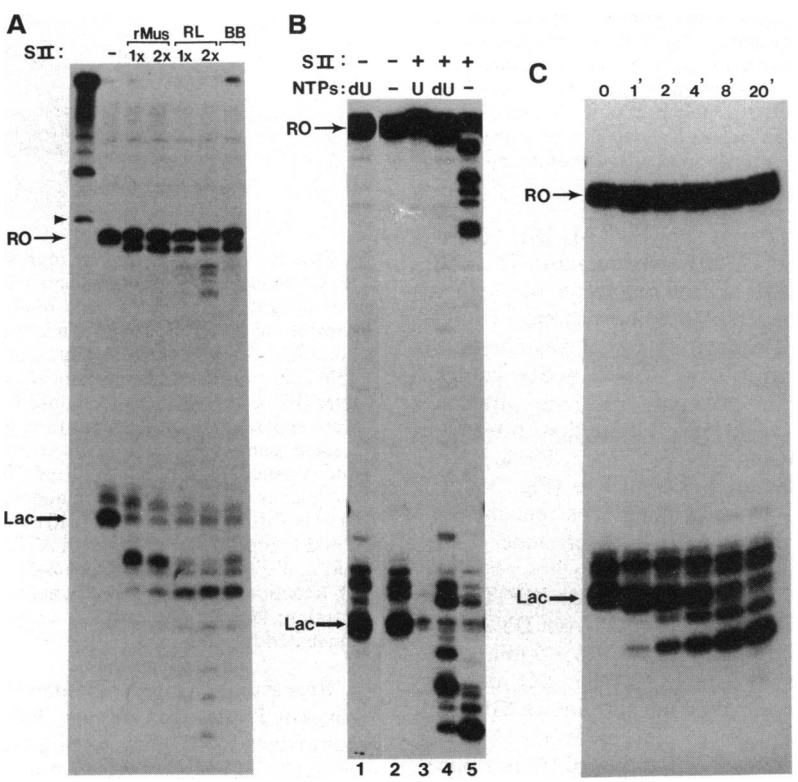

FIG. 5. (A) Repressor-arrested complexes cleave their RNAs. Washed elongation complexes were made 7 mM in $MgCl_2$. Buffer (−), recombinant mouse SII (rMus; x = 8 μg), rat liver SII (RL; x ≈ 100 ng of phenyl fraction), or bovine brain SII (BB; 2 μg) were added and incubated for 30 min. Arrowhead indicates 260-nucleotide marker RNA. (B) Trapping of cleaved RNA. Washed elongation complexes (lane 2) were made 7 mM in $MgCl_2$. Pure calf thymus SII (≈290 ng; lanes 3–5) or buffer (lanes 1 and 2) was added with ATP, GTP, CTP (800 μM each; lanes 1, 3, and 4), and UTP (800 μM; lane 3), 3′-dUTP (Boehringer Mannheim; 1.7 mM; lanes 1 and 4), or no NTPs (lane 5) and incubated for 30 min. (C) Time course of transcript cleavage. Calf thymus SII (≈100 ng), $MgCl_2$ (7 mM), ATP, GTP, CTP (800 μM each), and 3′-dUTP (1.7 mM) were added to washed complexes, and the mixtures were incubated for the indicated times (min).

RNAs did not become extended after 30 min in a nuclear extract. In our system little or no termination takes place, since repressor-arrested complexes were isolated and activated by SII for readthrough. This suggests that *in vivo* repressor blockade does not *necessarily* result in transcription termination. SII is found in numerous species and cell types, yet repressor bound to an intragenic site reduces gene expression in living cells (9). Thus, it seems probable that SII activity is under control *in vivo*. SII exists as a phosphoprotein (17), but this modification is not known to alter its function in transcription elongation. Little is known about changes in SII levels or activity *in vivo*. When added before RNA polymerase has reached repressor, SII does not abolish arrest at DNA-bound repressor, but it reduces the half-life of arrested complexes (J. M., unpublished data). Thus, *in vivo* the elongation complex may pause long enough to result in destabilization of the nascent transcript and, consequently, reduced gene expression.

We mapped the 3′ end of the arrested RNA and found that it is close to the DNase I boundary of *lac* repressor. This is similar to the results seen for *E. coli* RNA polymerase arrested by *lac* repressor (14, 15) or *Eco*RI (16), where RNA polymerase extends RNA chains to within a few base pairs of the blockading protein's DNase I footprint boundary. This suggests that the 3′ terminus of a growing RNA chain is also very close to the leading edge of RNA polymerase II.

Most importantly, this report shows that an elongation factor can play a role in enabling readthrough of a specific DNA-binding protein that efficiently blocks elongation. SII binds to RNA polymerase II and facilitates readthrough of arrest signals in naked templates (25, 27, 43) and nucleosome-enhanced pauses (7). Thus, SII functions generally to allow RNA polymerase II to transcribe through different kinds of impediments. We also show that SII activates nascent transcript cleavage in repressor-arrested elongation complexes, strengthening the idea that RNA cleavage is an integral part of SII function.

Repressor blockage is probably relieved by the eventual departure of repressor from DNA. Factors that displace other DNA-bound proteins have been described. The mechanisms by which they act vary. For λ repressor, monomer proteolysis results in the displacement of dimers from DNA (44). Proteins can also be displaced from DNA by a helicase (45). RNA polymerase-binding proteins can reduce the DNA-binding potential of RNA polymerases (46, 47). Similarly, transcription factors can complex with proteins that prevent them from binding DNA (48). We did not find that SII directly antagonized *lac* repressor DNA binding. Instead, SII activated a latent nuclease activity of the arrested elongation complex. SII may function in repressor-readthrough by moving arrested RNA polymerase away from a transcriptional impediment, enabling chain reextension and eventual readthrough (30).

lac repressor, but not all DNA-binding proteins (e.g., TTFI), blocks transcript elongation by RNA polymerase II (8, 9). Specific protein–protein interactions between RNA polymerase I and TTFI may mediate its polymerase-specific function (8). A DNA-binding protein that terminates transcription is also found in mitochondria (49, 50). Two DNA-binding proteins, a CCAAT-box protein (51) and polyomavirus T antigen (52), block transcription *in vivo* by RNA polymerase II. It will be interesting to learn which DNA-binding proteins in eukaryotes have functionally significant RNA polymerase II contact surfaces and if SII can provide readthrough for those that block transcription.

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