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In Vitro Activity of the Baculovirus Late Expression Factor LEF-5

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The baculovirus late expression factor LEF-5 has a zinc ribbon that is homologous to a domain in the eukaryotic transcription elongation factor SII. To determine whether LEF-5 is an elongation factor, we purified it from a bacterial overexpression system and added it to purified baculovirus RNA polymerase. LEF-5 increased transcription from both late and very late viral promoters. Two acidic residues within the zinc ribbon were essential for stimulation. Unlike SII, however, LEF-5 did not appear to enable RNA polymerase to escape from intrinsic pause sites. Furthermore, LEF-5 did not increase transcription in the presence of small DNA-binding ligands that inhibit elongation in other systems or viral DNA-binding proteins which inhibit the baculovirus RNA polymerase. Exonuclease activity assays revealed that baculovirus RNA polymerase has an intrinsic exonuclease activity, but this was not increased by the addition of LEF-5. Initiation assays and elongation assays using heparin to prevent reinitiation indicated that LEF-5 was active only in the absence of heparin. Taken together, these results suggest that LEF-5 functions as an initiation factor and not as an elongation factor.

The transcription of baculovirus late genes requires 19 different viral-encoded proteins, called LEFs (late expression factors). One of these is LEF-5, which has significant sequence homology to the C-terminal domain of the eukaryotic transcription factor SII (also known as TFIIS). SII is an elongation factor, but unlike other elongation factors, it does not stimulate the rate of transcription elongation (reviewed in reference 34). Rather, it increases the ability of RNA polymerase II (Pol II) to synthesize long RNA transcripts (13, 28, 33). It does so by restarting Pol II after it becomes arrested. In the arrested state, Pol II is still engaged in a ternary complex but is unable to extend, possibly because the active site has lost contact with the 3′ end of the RNA. SII stimulates the RNA cleavage activity of Pol II, which produces a new 3′ OH within the active site (17, 20, 27). Cleavage of nascent transcripts allows a stalled polymerase to back up and attempt to read through blockages repeatedly until it successfully extends beyond the arrest site (13, 29).

SII contains three major structural domains, an N-terminal domain of unknown function, a middle domain that binds to RNA Pol II, and the C-terminal zinc ribbon that binds to nucleic acids (1). The zinc ribbon domain of SII contains several residues that are essential for function (15, 24, 25). Four invariant cysteine residues chelate zinc and form the ribbon structure. An Asp-Glu dipeptide in the loop of the ribbon participates in metal binding within the RNA Pol II active site, and a conserved tryptophan or phenylalanine interacts with RNA through stacking interactions.

LEF-5 proteins from 11 different baculoviruses have been sequenced, and all of these proteins contain residues capable of forming a C-terminal zinc ribbon with a central Asp-Glu dipeptide (10). But the LEF-5 proteins also have several sequence features that differ from those of the SII consensus. Both the first loop and the first β sheet are shorter in LEF-5 than in SII, although a computer modeling study by Harwood et al. (10) predicted that the C-terminal domain of LEF-5 could fold into a zinc ribbon structure similar to that in SII. In addition, LEF-5 has a histidine in place of the second cysteine. While this should not affect the ability of the ribbon to bind zinc, histidine substitutions have not been identified in any of the 25 or more SII proteins sequenced from diverse eukaryotic sources. Finally, all of the LEF-5 proteins lack the conserved aromatic residue in the zinc ribbon. This residue is essential for SII function, so LEF-5 must use other residues to bind RNA if it functions in a manner similar to SII.

The goal of the present study was to determine whether LEF-5 functions as a transcription elongation factor. To this end, we purified LEF-5 from a bacterial overexpression system and examined its activity in transcription reactions containing purified baculovirus RNA polymerase. We found that LEF-5 strongly stimulated transcription activity but were unable to demonstrate that it stimulated the cleavage activity of the viral RNA polymerase

MATERIALS AND METHODS

Construction of pCYB-LEF5. The LEF-5 open reading frame was amplified by PCR using *Pfu* DNA polymerase and the *Autographa californica* nuclear polyhedrosis virus genomic clone *Pst*I-C. The upstream primer (GAATTCCAT ATGTCGTTTG ATGATGGCGT CGTTAAGGCG) inserted an *Nde*I site (underlined) at the AUG codon, and the downstream primer (CTGCAGCGCTCT TCCGCAAGAACCAGACATTCC) inserted a *Sap*I site (underlined) after the last amino acid encoded by LEF-5. The 822-bp PCR product was purified on a 1% low-melting-point agarose gel and then digested with *Nde*I and *Sap*I. The resulting fragment was cloned into the intein expression vector pCYB1, which had been digested previously with *Nde*I and *Sap*I. The correct LEF-5–intein expression vector, called pCYB-LEF5, was identified by restriction digestion and confirmed by DNA sequence analysis.

Site-directed mutagenesis. Mutagenesis of the Asp-Glu dipeptide was done using the QuikChange site-directed mutagenesis protocol (Stratagene). The primer DE248/249AA (GTCGAAAGCC AAACGAGGGC AGGCGCCGCA ATTGCTTCGT TCATTCGCTA CTGTCGGCTG) and its complement were used for amplification of pCYB-LEF5. The primer pair was designed to intro-

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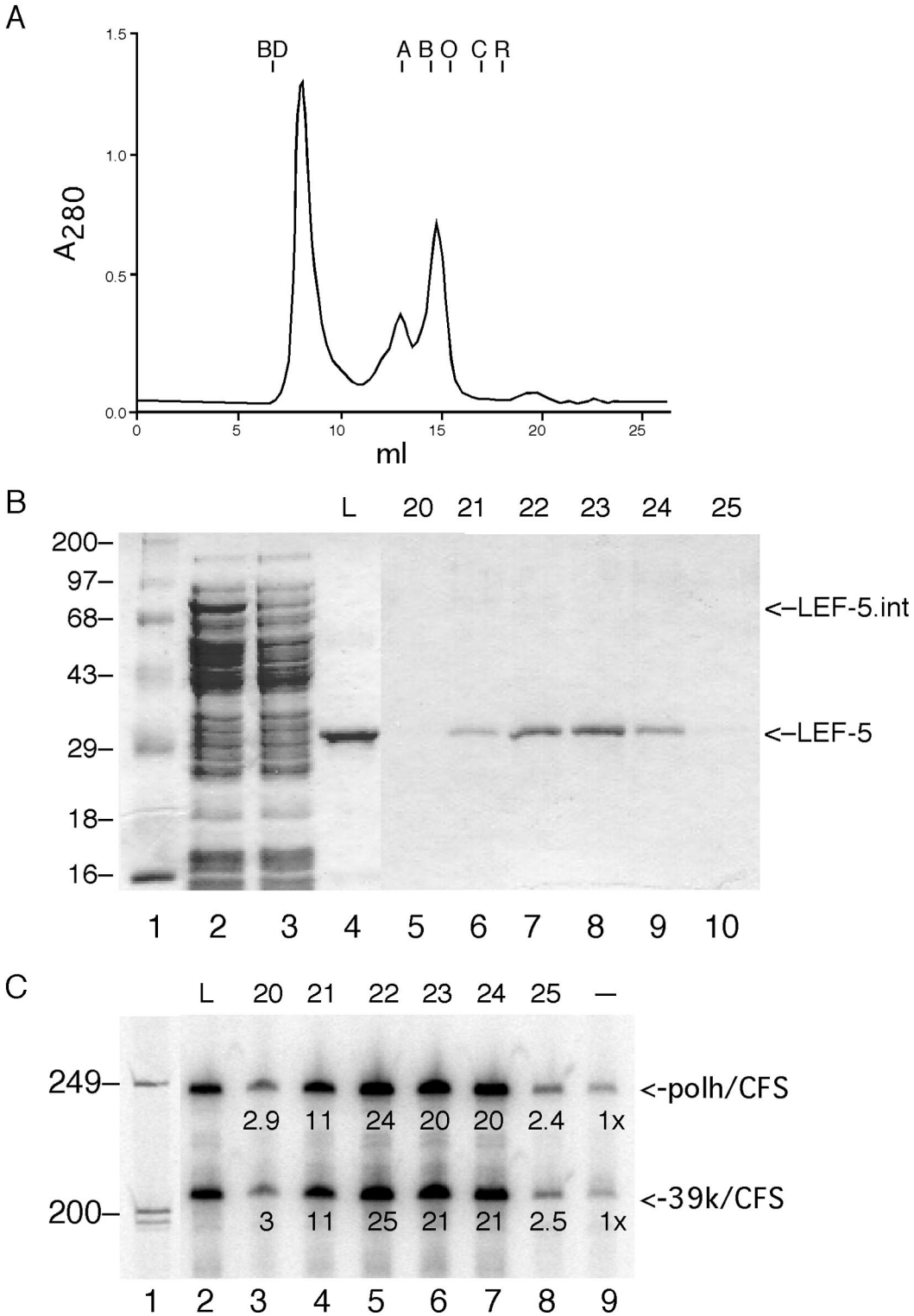


FIG. 1. Purification of LEF-5. (A). Gel filtration of LEF-5. LEF-5 was expressed in bacteria, and total soluble protein was loaded onto a chitin column. The flowthrough was discarded, and bound proteins were eluted after an overnight incubation in 30 mM DTT. Partially purified LEF-5 was then filtered through Superdex 200. Fractions (0.5 ml) were collected. The marker proteins used for calculation of the molecular weight of LEF-5 were aldolase (A) (molecular weight, 158,000), bovine serum albumin (B) (molecular weight, 67,000), ovalbumin (O) (molecular weight, 43,000), chymotrypsin (C) (molecular weight, 25,000), and RNase A (R) (molecular weight, 13,700). The exclusion volume was determined by gel filtration of blue dextran 2000 (BD). (B). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: 1, protein molecular weight markers, with molecular mass (in kilodaltons) of relevant proteins shown on the left; 2, total soluble protein; 3, chitin column flowthrough; 4, DTT eluate from chitin column which was loaded onto Superdex (L); 5 to 10, Superdex fractions 20 to 25, respectively, surrounding the LEF-5 peak. LEF-5 and LEF-5–intein fusion protein (LEF-5.int) are indicated on the right. (C). Transcriptional activity of LEF-5. Fractions were individually dialyzed

duce a silent mutation for restriction enzyme screening of potential recombinants with *Mfe*I (underlined) in addition to the alanine substitutions. Clones with the expected restriction digest pattern were verified by DNA sequencing analysis.

Purification of LEF-5. pCYB-LEF5, or one of the mutant versions of this construct, was transformed into BL21 cells, and a 500-ml culture was grown at 30°C in the presence of 50 μM $ZnCl_2$. Expression of LEF-5 was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by incubation at 20°C overnight. Bacteria were lysed by sonication in buffer 5 (50 mM Tris [pH 7.9], 1 M NaCl, 0.1 mM EDTA, 50 μM $ZnCl_2$), and soluble proteins were loaded onto a 1.5-ml (bed volume) chitin column. Contaminating proteins were removed by extensive washing in buffer 5. The fusion protein was allowed to self-cleave by overnight incubation in buffer 5 plus 30 mM dithiothreitol (DTT). Eluted LEF-5 was then further purified by filtration through a Superdex 200 (HR10/10) column. Fractions (0.5 ml) were collected, individually dialyzed against LEF-5 storage buffer (50 mM Tris [pH 7.9], 400 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50 μM $ZnCl_2$), and stored at −20°C. All of the experiments presented in this paper were repeated with at least two different preparations of polymerase under conditions in which LEF-5 stimulated transcription 10- to 35-fold in the absence of heparin.

In vitro transcription assays. Baculovirus RNA polymerase was purified from *Spodoptera frugiperda* cells infected with a mixture of four viruses, each expressing one subunit of RNA polymerase, as previously described (8, 16). Assays were performed using nucleoside-free cassettes linked to either the very late polyhedrin promoter pPolh/CFS or the late *39k* promoter p39kL/CFS as previously described (35). Transcription reactions lacked CTP, and elongation complexes stalled when they reached the first cytosine residue in the nontemplate strand. Transcription reactions contained 1 pmol of template, 0.2 pmol of baculovirus RNA polymerase, and 1 pmol of LEF-5 unless otherwise indicated. The final concentration of Zn in all assays was 10 μM, which is not inhibitory to baculovirus RNA polymerase (data not shown). PP31 was purified as described elsewhere (6).

Exonuclease assay. Stalled elongation complexes were purified through two successive Micro BioSpin-30 columns previously equilibrated in transcription buffer lacking $MgCl_2$. The reaction mixtures were then incubated at 30°C with the addition of 200 μM concentrations of nucleoside triphosphates (NTPs) and 2 mM $MgCl_2$, and 1 pM LEF-5 as indicated.

Construction of transcription templates. The clone pPolh/CFS-ADA contains the intron I arrest site of the murine adenosine deaminase gene (12, 18). It was constructed by annealing the 36-nucleotide (nt) oligonucleotide ADA.1 (GTA AGCACCG AGGGGCTCCG TTGCCAGGG TTCTGTC) and its complement and ligating with *Swa*I-digested pPolh/CFS-Swa2 (16). Potential clones were screened by DNA sequence analysis, and one clone with the oligonucleotide inserted into the proper orientation was selected for further experimentation. The clone pPolh/CFS-Adterm contains a well-characterized RNA Pol II arrest site inserted in the *Swa*I site of pPolh/CFS-Swa2. The clone pAd-Term-2 (31) was digested with *Taq*I, followed by repair of sticky ends with the Klenow fragment of *Escherichia coli* DNA polymerase I. The resulting 285-bp fragment containing the histone arrest site was purified by agarose gel electrophoresis and cloned into pPolh/CFS-Swa2. Potential clones were screened by restriction digest and confirmed by DNA sequence analysis.

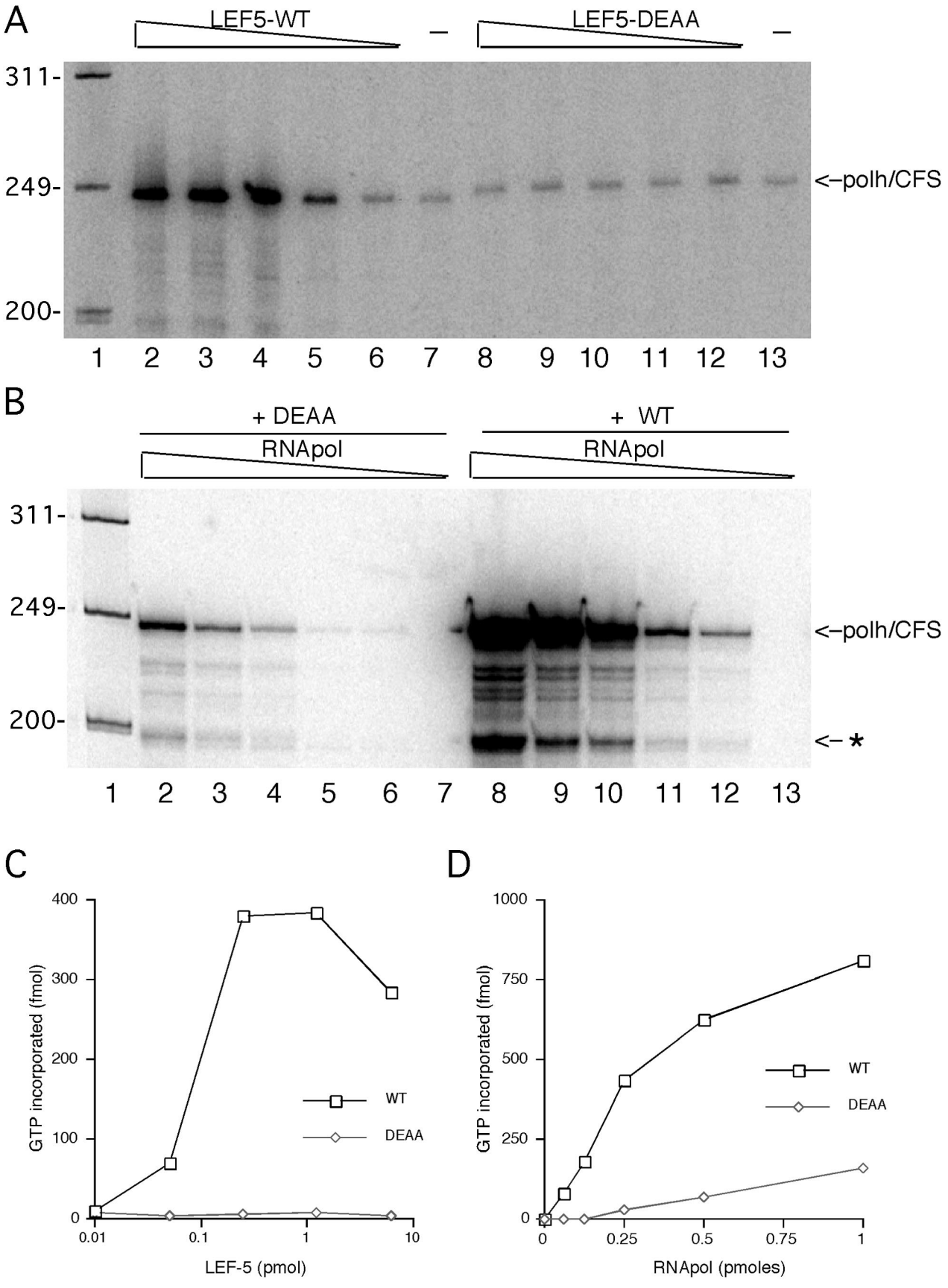
RESULTS

Purification of LEF-5. The LEF-5–intein fusion was overexpressed in BL21 cells and purified on a chitin column (Fig. 1B, lanes 2 to 4). The chitin column resulted in a high degree of purification, and no other proteins were visible by Coomassie blue staining. However, we have found that contaminants eluting from chitin columns nonspecifically bind to DNA and can inhibit transcription (data not shown). Therefore, it was necessary to further purify LEF-5 to remove this material. LEF-5 aggregates at physiological salt concentrations, so it was not possible to purify it by ion-exchange chromatography. Therefore, gel filtration through Superdex 200 at 1 M KCl was used for the final purification step (Fig. 1A). One UV-absorbing peak eluted near the void volume. These fractions contained the contaminating material that binds DNA but lacks detectable protein (data not shown). A second large peak eluted at 14.86 ml, and corresponding fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A protein with a molecular weight of approximately 31,000 was observed in the Coomassie brilliant blue-stained gels (Fig. 1B, lanes 5 to 10). Comparison of the elution volume for LEF-5 relative to those for marker proteins filtered through the same column indicated a molecular size of 59.6 kDa for the native protein. Assuming that the protein is roughly globular in shape, this result suggests that the LEF-5 protein with a molecular weight of 31,000 forms a dimer that is stable even with 1 M NaCl. Human SII is also a dimer in solution (38), as is the baculovirus RNA polymerase (8).

Fractions across the peak of protein were evaluated using our standard in vitro transcription assay for the ability to stimulate purified baculovirus RNA polymerase (Fig. 1C, lanes 3 to 8). This assay uses two nucleoside-free templates that are separately linked to the late *39k* gene and the very late polyhedrin (*polh*) gene (35). A 2-μl aliquot of each Superdex 200 fraction was added to a constant amount of RNA polymerase. After incubation at 30°C for 12 min, samples were extracted with phenol and RNA products were analyzed on acrylamide gels in the presence of 8 M urea. As previously reported, the baculovirus RNA polymerase catalyzes the template-dependent synthesis of transcripts initiating at the baculovirus late promoters (8). Transcript levels were higher in the reactions containing LEF-5, and the level of increase was proportional to the amount of LEF-5 in each fraction, indicating that LEF-5 was responsible for the stimulation. Transcripts from the *39k* and *polh* promoters were increased equivalently, consistent with the idea that LEF-5 functions as an elongation factor and therefore its activity is not affected by promoter sequence.

Activity of mutant versions of LEF-5. To confirm that LEF-5, not contaminating proteins, was responsible for the increased transcription activity, we also constructed and tested three mutant versions of the protein. The acidic dipeptide Asp-Glu in the zinc ribbon of SII has been shown to be essential for the activity of SII (15). Therefore, these residues were targeted for alanine-substituted mutagenesis. A mutant version of LEF-5, containing a substitution of alanine for glutamic acid 248 (E248A), aspartic acid 249 (D249A), or both (DEAA), was purified in the same manner as was the wild-type version of the protein. The activity of LEF-5 was then determined by titration of in vitro transcription reactions (Fig. 2A). The wild-type version of the protein strongly stimulated transcription at submicromolar concentrations (Fig. 2C). The addition of 0.25 pmol of LEF-5 (Fig. 2A, lane 4), a concentration approxi-

against LEF-5 storage buffer, and then a 2-μl aliquot was added to standard in vitro transcription reaction mixtures containing pPolh/CFS and 39k/CFS. The results were quantitated by phosphorimager analysis. Lanes: 1, positions of relevant ϕX174-*Hin*fI molecular size markers in bases; 2, Superdex load (L); lanes 3 to 8, Superdex fractions 20 to 25, respectively; 9, LEF-5 storage buffer. The positions of the transcription products are indicated on the right. The levels of stimulation (*n*-fold) of the very late polh/CFS transcript and the late 39k transcript are indicated below the relevant transcripts.



mately equal to that of the baculovirus RNA polymerase, resulted in a 29-fold increase in transcription compared with that in reactions lacking LEF-5. When the concentration was increased by an additional fivefold, transcription was not increased, suggesting that RNA polymerase was limiting. An additional fivefold increase in LEF-5 concentration resulted in slight inhibition of activity. The LEF-5(DEAA) mutant did not stimulate transcription at any of the concentrations tested. The two single mutant proteins also lacked the ability to stimulate transcription (data not shown).

We then titrated RNA polymerase at a constant saturating amount of LEF-5 (Fig. 2B). In the absence of LEF-5 (data not shown) or in the presence of LEF-5(DEAA) (Fig. 2B, lanes 2 to 7), the transcription activity was directly proportional to the amount of RNA polymerase added to the reaction. The addition of LEF-5 stimulated transcription 30-fold at the lower concentrations of RNA polymerase but only sevenfold at the highest concentration tested. It was not possible to test higher concentrations of RNA polymerase because glycerol from the storage buffer would begin to inhibit the reaction (data not shown).

In this experiment, only the template pPolh/CFS was used so that we could also monitor the presence of a truncated transcript that is frequently observed in these reactions (Fig. 2B). This is the only shorter product that is consistently seen with this template, and Xu et al. previously speculated that it may be a pause product (35). The addition of LEF-5 did not decrease the intensity of this band relative to that of the full-length product. The ratio of truncated to full-length product was essentially constant (35 to 40% of the products were truncated) in all reactions with or without LEF-5. This suggests that the increase in transcription was not due to an increase in the ability of RNA polymerase to read through this block to transcription.

Transcription through RNA Pol II pause sites. Although LEF-5 did not promote read-through of the putative pause site within polh/CFS, it seemed premature to conclude that it was not capable of reactivating arrested RNA polymerase. This site has not been characterized, and it is not known for certain whether the truncated products arose from paused polymerases. In the field of RNA Pol II research, most studies of the elongation factor SII have used the plasmid pAd-Term2, which contains the extensively characterized arrest site of a human histone gene linked to the adenovirus major late promoter (27). In addition, this site effectively arrests *E. coli* RNA polymerase, which can be released by the bacterial elongation factors GreA/GreB (22). Therefore, we constructed a transcription template containing this arrest site linked to the polyhedrin promoter to test the effect of LEF-5 on templates paused at this site (Fig. 3A). A 285-nt restriction fragment containing two strong pause sites was cloned into the transcription template pPolh/CFS. The point of insertion was 88 nt from the 3′ end of the C-free region of this template.

In the absence of CTP, transcripts arising from pPolh/CFS-AdTerm are shorter than those from the parental pPolh/CFS because the histone arrest site fragment contains cytosine residues (Fig. 3B, compare lanes 2 and 8). Furthermore, it is important to note that the bands in lane 8 represent elongation complexes that were artificially stalled before transcription of the histone sequences due to the lack of CTP in the reaction. Stalled complexes were released by the addition of 1 mM CTP to allow transcription through this histone region and beyond the C-free region. At the same time, 1 mM cold GTP was added to prevent further incorporation of label. The fully extended products were very long and migrated near the top of the gel because the plasmids were circular. With pPolh/CFS-AdTerm, several bands were observed that correlated with pausing within the histone cassette. The addition of LEF-5, however, had no effect on the intensity of these bands. This indicates that LEF-5 did not increase the ability of RNA polymerase to transcribe beyond these arrest sites.

A second template, which contained an oligonucleotide corresponding to the stable transcription arrest site in the murine adenosine deaminase gene, was constructed (18). The transcription of this template, called pPolh/CFS-ADA, also produced products in the region where paused elongation complexes are expected. This arrest site, however, did not efficiently block RNA polymerase, as most of the transcripts were fully extended. The addition of LEF-5 had no effect on transcription through this region. Numerous truncated products that represent products stalled or arrested at positions beyond the C-free cassette were also observed, as was also the case with pPolh/CFS. It is not clear whether these represent true arrest sites, as the pattern of bands differed from experiment to experiment (compare Fig. 3B, lanes 2 and 3, and Fig. 5A, lanes 2 and 9). Again, LEF-5 had no effect on the ability of RNA polymerase to transcribe beyond these sites.

Fig. 3C shows the results of a control reaction demonstrating that the concentration of LEF-5 used in this experiment is capable of producing a 34-fold stimulation of activity when added to the reaction initially. The lanes shown are underexposed relative to those in Fig. 3B to better represent the difference between the lanes. Taken together, these results suggest that LEF-5 does not exert its activity by increasing the ability of RNA polymerase to read through intrinsic pause sites.

Transcription termination. The RNA Pol II arrest sites frequently map to sequences containing thymidine residues on

FIG. 2. Titration of LEF-5 and baculovirus RNA polymerase. (A) LEF-5 titration. Wild-type (WT) LEF-5 or the LEF-5(DEAA) mutant was added to a constant amount of RNA polymerase. The amount of LEF-5 added was varied from 0.01 to 6.2 nmol in fivefold increments [(lanes 2 to 6 show the results with decreasing amounts of WT LEF-5, and lanes 8 to 12 shown the results with decreasing amounts of LEF-5(DEAA)]. An equivalent volume of LEF-5 storage buffer was added to control reactions (lanes 7 and 13). Lane 1, ϕX174-*Hin*fI molecular size markers in bases. The template was pPolh/CFS. (B) RNA polymerase titration. Purified RNA polymerase was added to 1 pmol of WT LEF-5 or the LEF-5(DEAA) mutant. The concentration of RNA polymerase was varied from 1 to 0.6 pmol in twofold increments [lanes 2 to 7 for LEF-5(DEAA) and lanes 8 to 13 for WT LEF-5]. Lane 1, ϕX174-*Hin*fI molecular size markers in bases. (C) The data presented in panel A were quantitated using a Molecular Dynamics Storm phosphorimager and ImageQuant software. (D) The data presented in panel B were quantitated by phosphorimager analysis.

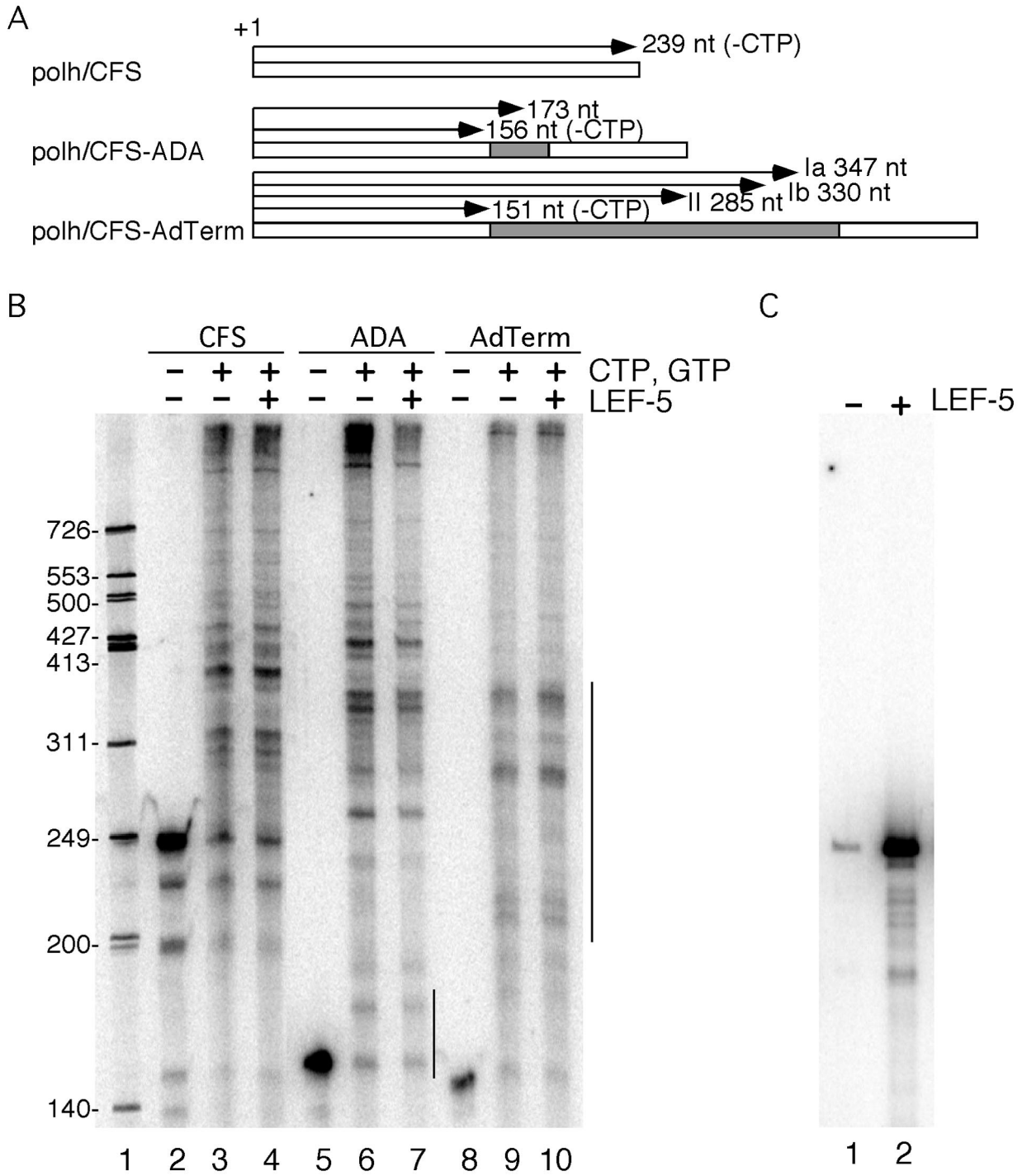


FIG. 3. Pause sites. (A) Schematic diagram of transcription templates. (B) Transcription through Pol II pause sites. Transcription reactions were performed with the standards pPolh/CFS (lanes 2 to 4), pPolh/CFS-ADA (lanes 5 to 7), and pPolh/CFS-AdTerm (lanes 8 to 10). After 12 min at 30°C, reactions were stopped and analyzed directly (lanes 2, 5, and 8), chased with 1 mM CTP and GTP and LEF-5 buffer (lanes 3, 6, and 9), or chased with 1 mM CTP, GTP, and 0.3 nM LEF-5 (lanes 4, 7, and 10). Lane 1 contains ϕX174-*Hin*fI markers, with bases indicated on the left. Vertical bars represent the positions of expected pause products. (C) Transcription in the presence (lane 2) and absence (lane 1) of LEF-5. A set of control reactions was performed to show that LEF-5 stimulated transcription when added at the same time as RNA polymerase.

the nontemplate strand sites (34). It has previously been shown that baculovirus RNA terminates transcription within T-rich sequences (16). The products appear to be terminated and not merely stalled or arrested because they are also polyadenylated. But in most systems, termination of transcription follows pausing of the elongation complex (2, 9, 19, 37). Therefore, we wanted to determine whether the addition of LEF-5 affected the frequency of termination and polyadenylation on the tem-

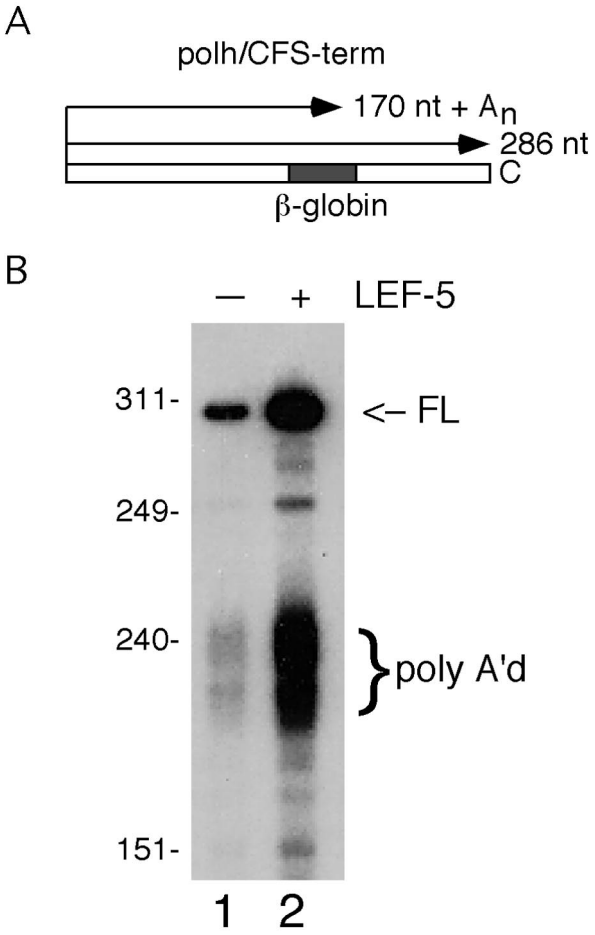


FIG. 4. Termination and polyadenylation. (A) Schematic diagram of transcription templates and sizes of expected products. (B) Transcription reaction. Purified RNA polymerase (0.2 pmol) was incubated with 0.2 pmol of polh/CFS-T under standard transcription reaction conditions at 30°C for 15 min in the presence (lane 2) or absence (lane 1) of LEF-5. Lane 1 shows the positions of relevant ϕX174-*Hin*fI markers, with bases indicated on the left. The positions of full-length C-free product (FL) and the terminated polyadenylated products (poly A′d) are indicated on the right.

plate pPolh/CFS-Term. As previously described, this template contains a 44-nt globin cleavage and polyadenylation sequence and baculovirus RNA polymerase terminates within a T-rich region of this template (Fig. 4A). Only half of the elongating complexes recognize this signal. The other complexes artificially stall upon reaching the first cytosine residue in the nontemplate strand (Fig. 4B). The addition of LEF-5 strongly stimulated the transcription reactions but had no effect on the relative amounts of full-length, terminated, or polyadenylated products.

Transcription through DNA-bound drugs. SII allows RNA Pol II to transcribe through small ligands bound to the minor groove of DNA (21). To test whether LEF-5 also demonstrated this activity, we determined the effect of adding distamycin and mithramycin to elongation complexes (Fig. 5). Transcription on pPolh/CFS was initiated in the absence of both drugs and LEF-5. In the absence of CTP, transcription was artificially halted at the first C residue. Elongation was then allowed to proceed by the addition of CTP along with cold GTP to prevent further labeling of RNA chains. In the absence of other factors, most complexes were fully extended and reached lengths greater than that of the largest molecular weight marker, likely representing transcription of the whole plasmid. To test the effect of the drugs, distamycin (Fig. 5A) or mithramycin (Fig. 5B) was added along with LEF-5 or LEF-5(DEAA) and CTP and GTP. Both drugs produced a reduction in the size of RNA transcripts in a dose-dependent manner. Several pause sites were observed, and each drug produced a distinctive set of shortened transcripts. At the highest concentrations tested, most transcription stopped within 200 nt of the unextended product. At lower drug concentrations, longer transcripts were synthesized. The addition of LEF-5 to the reactions had no effect. Essentially identical results were obtained with the wild-type version of LEF-5 and the mutant version of LEF-5, which was not capable of stimulating transcription.

Transcription through bound protein. SII also enables RNA Pol II to transcribe past DNA-bound protein (30). To test the ability of LEF-5 to catalyze this reaction, we performed transcription reactions in the presence of PP31 (Fig. 6). It has been shown elsewhere that PP31 binds to DNA and strongly inhibits in vitro transcription reactions (6). To test whether LEF-5 could relieve this inhibition, PP31 and pPolh/CFS were preincubated for 15 min on ice to allow for binding prior to the addition of the transcription mix. LEF-5 and baculovirus RNA polymerase were then added along with NTPs. Analysis of the in vitro products revealed that transcription was inhibited by PP31 in a concentration-dependent manner (Fig. 6A). The absolute transcription activity was higher in the reactions containing LEF-5 than in reactions with the DEAA version of the protein, but a plot of relative transcription levels as a function of PP31 concentration produced curves that were superimposable (Fig. 6B). This indicates that LEF-5 does not increase the ability of RNA polymerase to displace or read through protein bound to DNA.

Exonuclease activity of RNA polymerase. SII stimulates elongation by activating the intrinsic RNA cleavage activity of RNA Pol II. Cleavage of nascent transcripts allows a stalled polymerase to back up and attempt to read through blockages repeatedly until it successfully extends beyond the arrest site (13, 29). Therefore, it was of interest to determine whether baculovirus RNA polymerase has an intrinsic cleavage activity and whether LEF-5 was capable of stimulating this activity (Fig. 7). Transcription was initiated on pPolh/CFS, and artificially stalled complexes were purified by gel filtration to remove nucleotides. The complexes were then incubated for 10 min at 30°C in the presence or absence of $MgCl_2$, which is required for the cleavage reaction. In the absence of $MgCl_2$, complexes were stable for 10 min (Fig. 7, lanes 3 and 6) and, in a separate experiment, for 30 min (data not shown). In the presence of $MgCl_2$, cleavage of the nascent transcript was observed. The extent of cleavage was limited, and incubation for up to 30 min did not further decrease the size of the transcript (data not shown). The addition of LEF-5 had no effect on the cleavage activity, indicating that the cleavage activity was intrinsic to RNA polymerase. The addition of 500 μM concentrations of NTPs allowed full extension of the nas-

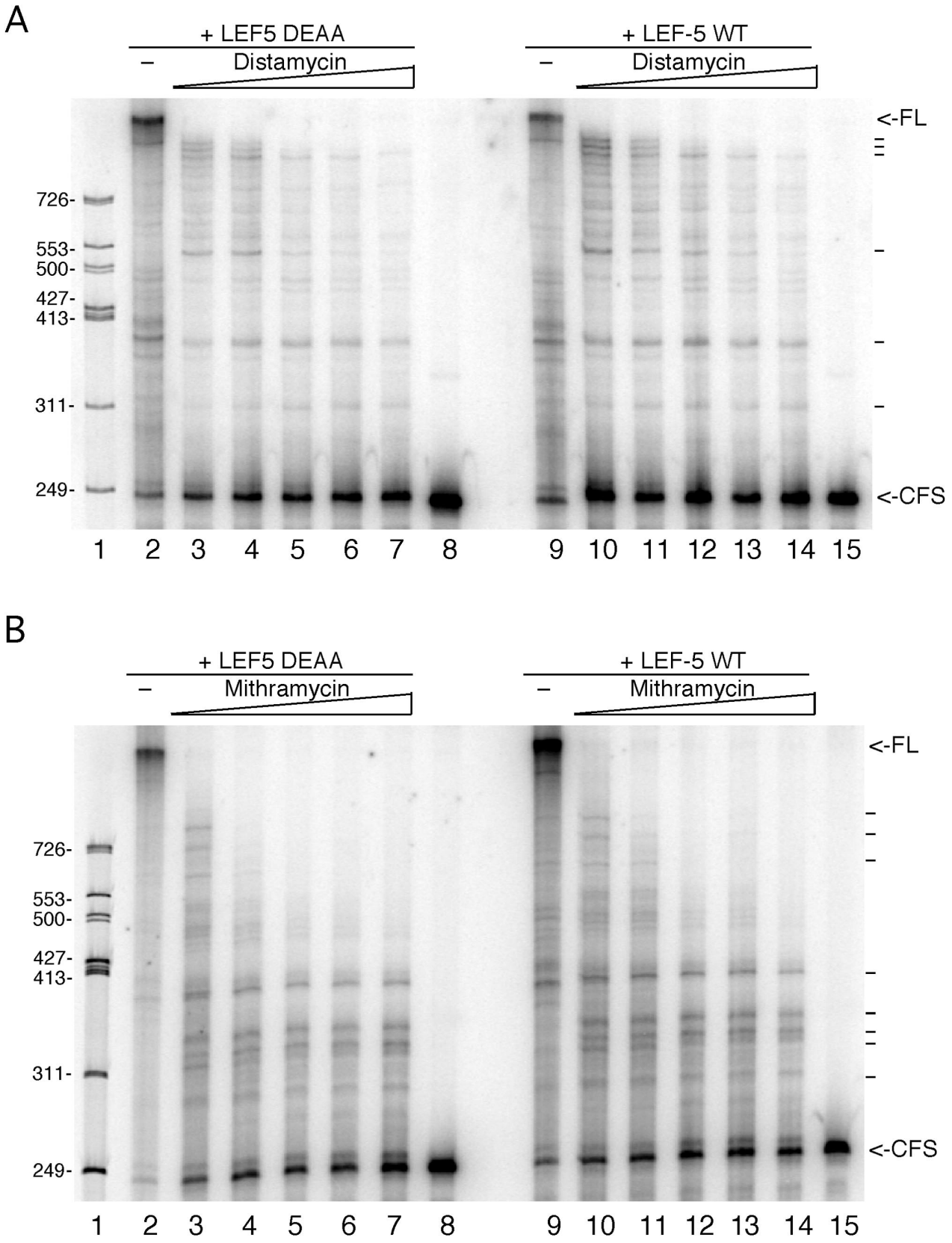


FIG. 5. Transcript elongation in the presence of ligands binding the minor groove of DNA. (A) Distamycin. Transcripts were initiated in the absence of CTP, ligands, and LEF-5. After 12 min at 30°C, distamycin was added to a final concentration of 0 mM (lanes 2, 8, 9 and 15); 15.62 μM (lanes 3 and 10); 31.25 μM (lanes 4 and 11); 62.5 μM (lanes 5 and 12); 125 μM (lanes 6 and 13); or 250 μM (lanes 7 and 14). Then, GTP and CTP were added to a final concentration of 250 μM (lanes 2 to 7 and 9 to 14) along with wild-type LEF-5 (WT) (lanes 9 to 15) or the LEF-5(DEAA) mutant (lanes 2 to 8). RNA was isolated after 30 min. (B) Mithramycin. The procedure was the same as that described for panel A. Lanes 1, ϕX174-*Hin*fI molecular markers in bases. FL, full-length C-free product; CFS, stalled transcript in the absence of CTP.

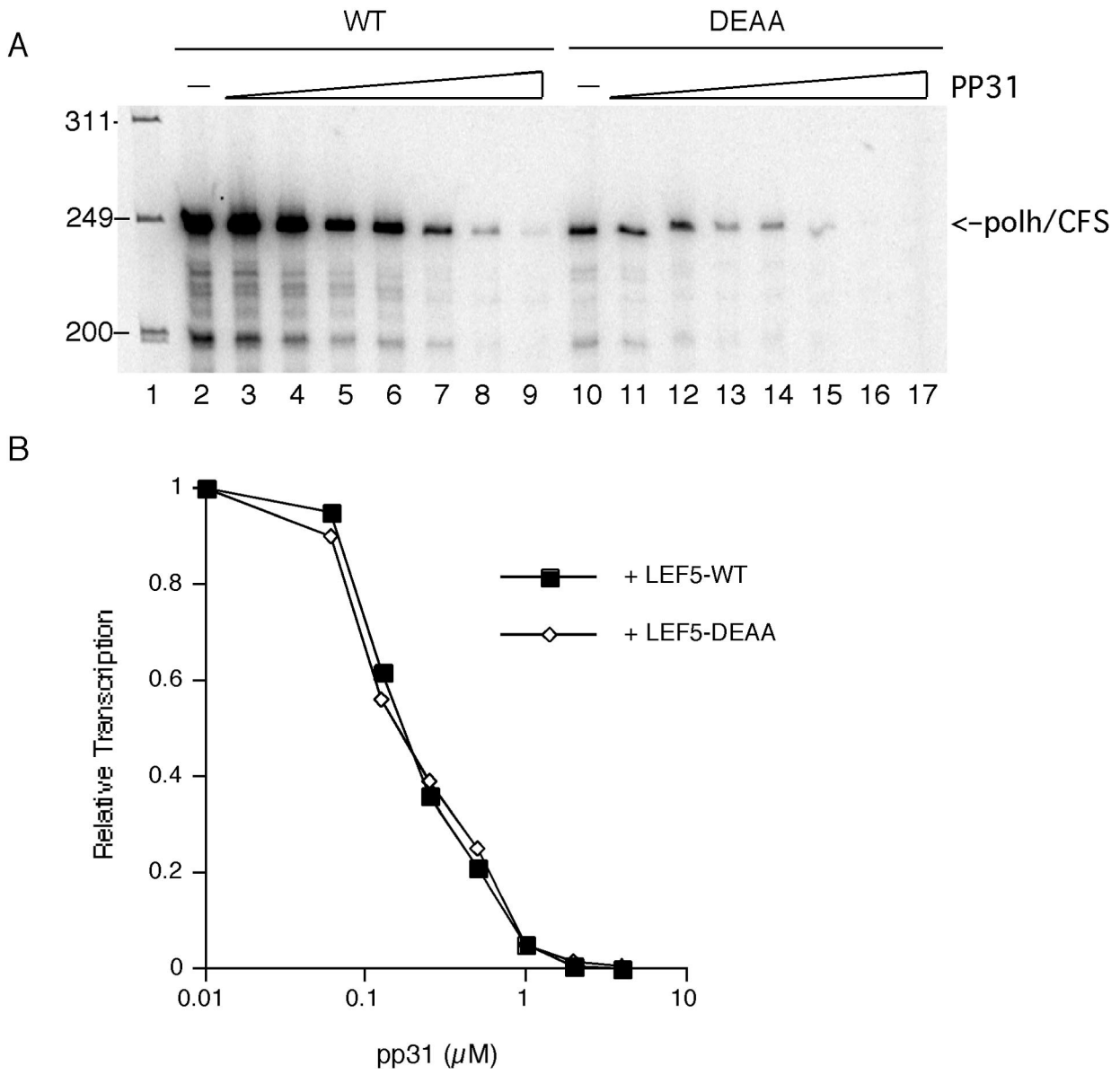


FIG. 6. Inhibition of transcription by PP31 is not relieved by LEF-5. (A) Transcription reactions. The concentration of polh/CFS was held constant at 0.1 μg/ml (15 μM concentration of nucleotide), and the concentration of PP31 was varied from 0.06 to 4 μM in twofold increments [lanes 3 to 9 for wild-type (WT) LEF-5 and lanes 11 to 17 for the LEF-5(DEAA) mutant]. PP31 was diluted in storage buffer containing bovine serum albumin, and the same buffer plus bovine serum albumin was added to the control reaction so that the buffer concentration and total protein amount were the same in every tube. WT LEF-5 or the LEF-5(DEAA) mutant was added at a final concentration of 6 nM. In vitro transcription assays were performed as previously described (8). Reaction products were separated on a 6 M urea, 6% polyacrylamide gel. Lane 1, ϕX174-*Hin*fI molecular size markers. (B) Quantitation. Reaction products were quantitated by phosphorimager analysis. The amount of product obtained relative to that in the absence of PP31 was plotted versus input PP31.

cent transcript, confirming that the ternary complexes were not inactivated by the treatment.

Transcriptional fidelity. One consequence of SII-induced cleavage is an increase in transcriptional fidelity, because SII stimulates the removal of misincorporated nucleotides (14, 32). To test whether LEF-5 can stimulate elongation in the presence of a chain terminator of RNA synthesis, we incubated stalled transcription complexes in the presence of 3′ dCTP (Fig. 8). With CTP alone, elongation complexes were able to fully extend and were able to synthesize long transcripts. In the presence of a twofold molar excess of 3′ dCTP, transcription was inhibited due to the incorporation of chain-terminating nucleotides. Reactions containing LEF-5 were indistinguishable from those lacking LEF-5, suggesting that LEF-5 did not stimulate proofreading.

Initiation assays. The results presented above strongly suggested that LEF-5 did not function in the same manner as SII. Therefore, we tested the activity of LEF-5 in assays that used heparin to inhibit initiation of RNA synthesis (Fig. 9A). First, we preincubated RNA polymerase with the template and

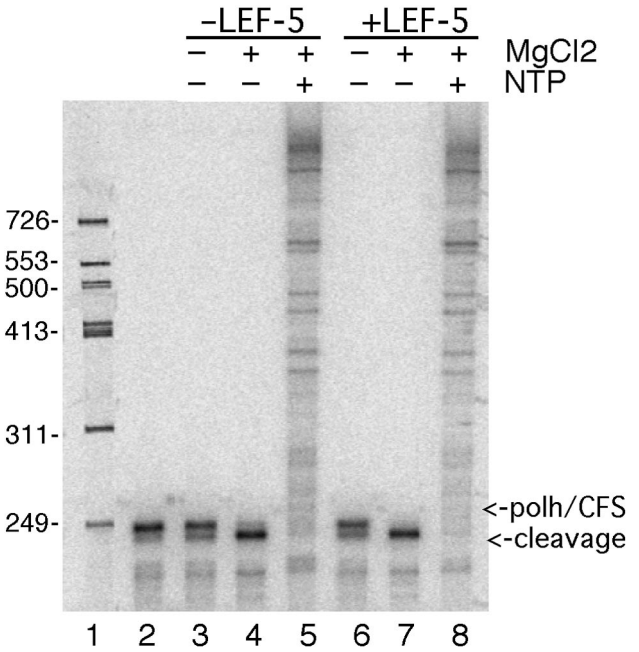


FIG. 7. Cleavage activity of the stalled elongation complex. Ternary complexes artificially stalled due to the absence of CTP were isolated by gel filtration (lane 2) and incubated in the presence (lanes 4, 5, 7, and 8) or absence (lanes 3 and 6) of $MgCl_2$ for 10 min at 30°C. As a control to demonstrate the elongation competency of the ternary complex after 10 min at 30°C, a 500 μM concentration of NTPs was added and incubation was continued for an additional 10 min. Lane 1, ϕX174-*Hin*fI molecular size markers.

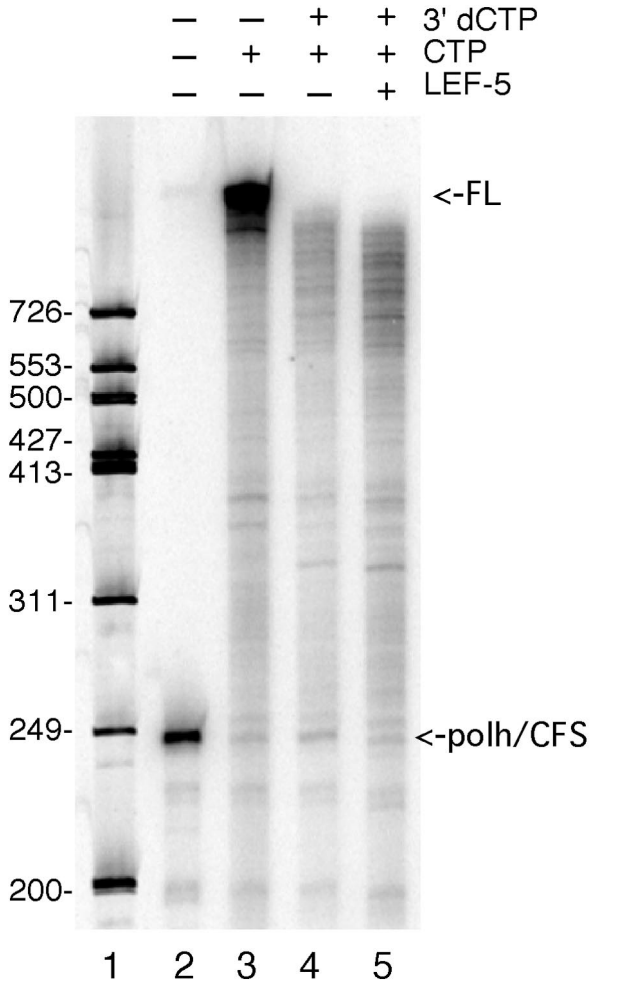


FIG. 8. dCTP inhibition. In vitro transcription reactions were stalled in the absence of CTP (lane 2) and then extended for 20 min in the presence of 500 μM CTP (lane 3) or 500 μM CTP plus 1 mM 3′ dCTP (lanes 4 and 5) and with (lane 5) or without (lanes 2 to 4) 1 pmol LEF-5. Lane 1, ϕX174-*Hin*fI molecular size markers.

LEF-5 to allow proteins and DNA to interact. We then added NTPs to initiate RNA synthesis, and after 1 min, heparin was added to inhibit further initiation. Funk et al. (5) have previously shown that heparin at a concentration of 10 μg/ml completely inhibits transcription, and we confirmed this in our system (data not shown). One minute of synthesis in the presence of LEF-5 produced a burst of synthesis that was 10-fold higher than in a parallel reaction containing the mutant version of the protein (Fig. 9A). At subsequent time points, the ratio of incorporated GTP in the presence of LEF-5 was 7- to 10-fold higher than with the mutant protein. This suggests that the effect of LEF-5 was limited to the time period before the addition of heparin.

To confirm this result, we tested the effect of adding LEF-5 to elongation complexes (Fig. 9B). The reactions were set up in a similar manner, except that LEF-5 was not added until 2 min after the addition of heparin. In this case, LEF-5 had no effect on the incorporation of GTP (note that the scales in Fig. 9A and B are different), and the curves for LEF-5 and LEF-5(DEAA) were superimposable. The results of these two experiments indicate that LEF-5 increases the number of elongation complexes but does not increase the rate of elongation or the ability of elongation complexes to make long transcripts.

DISCUSSION

The goal of our experiments was to determine whether the baculovirus late expression factor LEF-5 functioned in the same manner as the Pol II elongation factor SII. Harwood et al. (10) previously identified a region of the LEF-5 protein with homology to the zinc ribbon domain of SII. Furthermore, they demonstrated that deletion of the putative zinc ribbon decreased the ability of LEF-5 to stimulate late gene expression by 70%. A similar decrease was obtained with single point mutations of the zinc ligands and the acidic residues in the QTRxxDE loop. The remaining 30% of activity is significant, however, because in SII the zinc ligands and acidic loop residues are absolutely essential for activity and substitution of any one of these residues is not tolerated (4, 15). One explanation for these observations is that LEF-5 is multifunctional, with one activity that maps to the zinc ribbon domain and another that maps to the N-terminal part of the protein. Alternatively, these data could indicate that LEF-5 is not a functional homolog of SII.

The mutational analysis of LEF-5 conducted by Harwood et al. (10) was performed using a transient-expression assay. This is an in vivo assay that involves cotransfection and coexpression of 19 different viral genes and is not amenable to biochemical analyses of LEF-5 function. Therefore, we believed that the

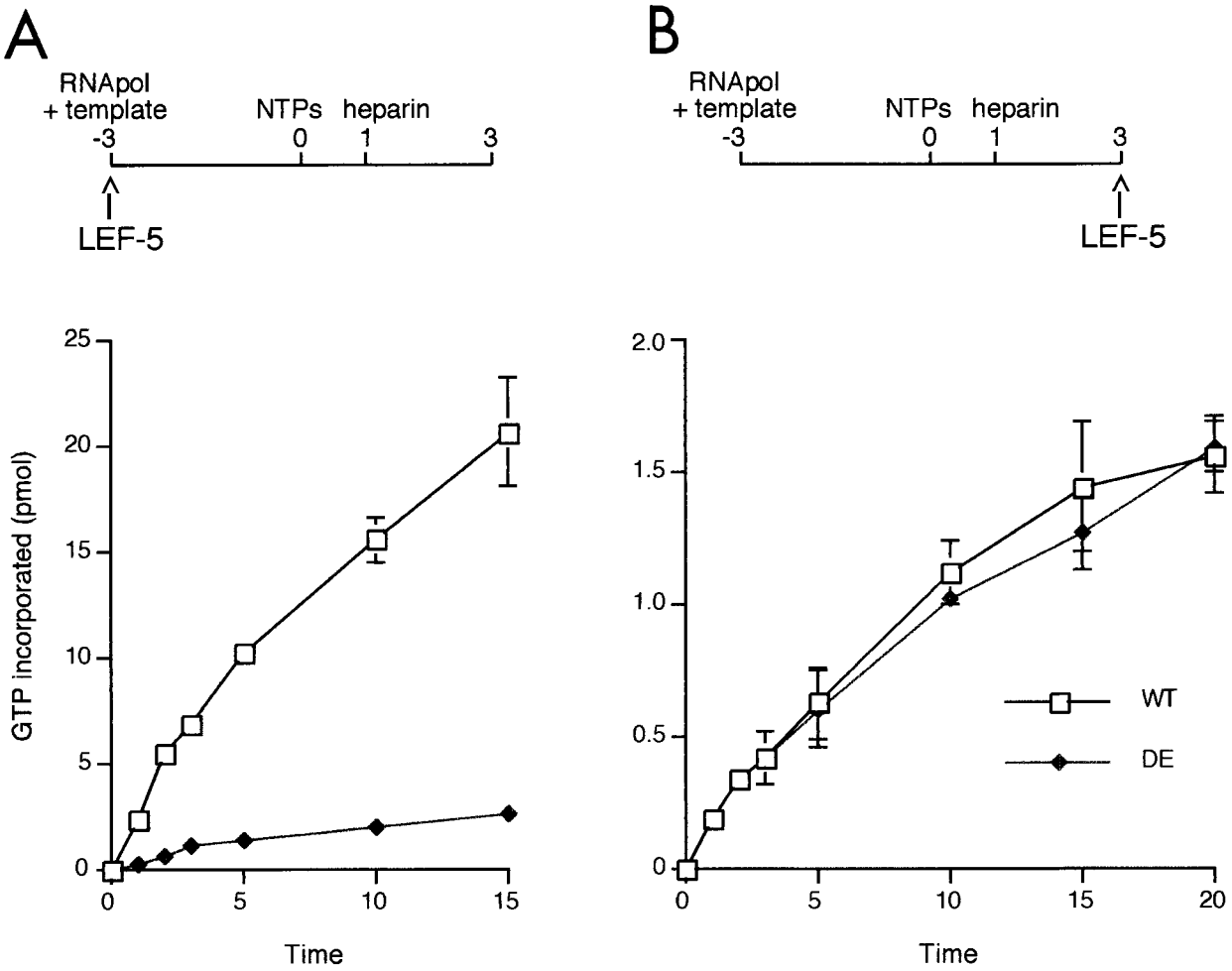


FIG. 9. Effect of LEF-5 on initiation and elongation. (A) Initiation reaction. Two 150-μl reaction mixtures containing 0.2 pmol of RNA polymerase, 1 μg of pPolh/CFS, and 1 pmol of LEF-5 or LEF-5(DEAA) were preincubated for 3 min. NTPs were added at time zero, and the reaction mixtures were incubated for another 1 min at 30°C. Then, 10 μg of heparin/ml was added to inhibit further initiation. Aliquots (12.5 μl) were removed at the indicated times. (B) Heparin-resistant elongation. The reaction conditions were identical to those described for panel A except that LEF-5 was added 3 min after the addition of NTPs. Data indicate means of triplicate reactions ± standard errors of the means.

question of LEF-5 function was better addressed by using an in vitro transcription system, where the role of LEF-5 in elongation could be tested directly.

Our results showed that LEF-5 strongly stimulated the transcription activity of baculovirus RNA polymerase. The transcriptions of both the late *39k* promoter and the very late polyhedrin promoter were equally stimulated by the inclusion of LEF-5 in transcription reactions. The promoter-independent nature of this stimulation suggests that LEF-5 does not act at the level of promoter recognition, although additional late and very late templates should be tested to confirm this. Furthermore, we showed that transcription stimulation was dependent on the acidic residues in the putative zinc ribbon. Two conclusions can be drawn from this result. One is that the stimulatory activity observed was due to LEF-5 and not to contaminating proteins in the LEF-5 preparation. The second is that the region of LEF-5 that is homologous to SII is essential for the transcription stimulation seen in vitro.

We performed a series of experiments to determine whether LEF-5 had the same mechanism of action as the eukaryotic elongation factor SII. First, we examined the production of a truncated transcript that is frequently produced from our standard C-free template. It is approximately 50 nt shorter than the transcripts that artificially stall at the first C on the nontemplate strand, and Xu et al. previously hypothesized that it was a transcriptional pause product (35). The addition of LEF-5 resulted in an increase in this product that was proportional to the increase in the full-length C-free product, indicating that LEF-5 did not increase the ability of RNA polymerase to transcribe through this region.

We then turned our attention to two well-characterized arrest sites for RNA Pol II. We cloned a fragment of a human histone gene, which has been used extensively to study the arrest of transcription by Pol II (31). This sequence also effectively arrests *E. coli* RNA polymerase, which can be released by the bacterial elongation factors GreA/GreB (22). The histone gene fragment posed a significant block to baculovirus RNA polymerase, although we did not demonstrate that the truncated products mapped to the same arrest sites as those for Pol II. The addition of LEF-5, however, did not alter the pattern of

pause products, indicating that LEF-5 was unable to reactivate polymerase after arrest at these sites. In addition, we tested an arrest site in the human adenosine deaminase gene (18). This appeared to present only a weak block to baculovirus RNA Pol II, as most transcripts extended through this region. Once again, however, LEF-5 had no effect on the appearance of pause products.

Next, we examined the ability of LEF-5 to enable RNA polymerase to transcribe past DNA-bound protein and DNA-bound drugs. As previously reported, the viral DNA binding protein PP31 binds template DNA and inhibits transcription by RNA polymerase (6). The addition of LEF-5 to transcription reactions increased overall transcription at all concentrations of PP31, yet the inhibition curves were superimposable. This indicates that the binding of PP31 to DNA has the same inhibitory effect on transcription, whether stimulated by LEF-5 or not. Similarly, we found that LEF-5 did not increase the ability of RNA polymerase to transcribe past the ligands binding the minor groove of DNA, mithramycin and distamycin (21).

Finally, we directly tested whether LEF-5 increased the exonuclease activity of RNA polymerase. This was done by incubation of washed ternary complexes in the absence of nucleotides and the presence of Mg^{2+}. Under these conditions, the exonuclease activities of RNA polymerases can be detected because the forward reaction, which is normally preferred, cannot proceed in the absence of nucleotides. The nuclease activities of Pol II and bacterial RNA polymerase are intrinsic yet strongly stimulated by SII or GreA/GreB (3, 11, 28, 33). We found that baculovirus RNA polymerase also possessed an intrinsic exonuclease activity, because the end of the nascent transcript was cleaved. Cleavage, however, was not enhanced by LEF-5. In addition, the extent of cleavage was limited to a single excision event, unlike the factor-enhanced cleavage seen with Pol II or *E. coli* RNA polymerase. The addition of elongation factors SII, GreA, and GreB to their respective polymerases results in a progressive shortening of nascent chains in dinucleotide or larger increments on artificially stalled complexes (13, 23). Furthermore, exonuclease activity was indirectly tested by incubation of stalled complexes in the presence of 3′ dCTP, a chain terminator of RNA synthesis. The concentration of the analog was sufficiently high so that extended synthesis would require excision of the chain terminators after incorporation. We found that the average length of products was not significantly higher in the presence of LEF-5.

The sum of the results of these experiments suggested that while LEF-5 stimulated transcription up to 35-fold in our assays, it did not increase the ability of RNA polymerase to synthesize long transcripts and thus did not function in the same manner as SII. Therefore, we examined the effect of LEF-5 on the formation of initiation complexes. We used heparin to inhibit reinitiation and therefore limited the assay to one round of transcription. We found that LEF-5 stimulated transcription 10-fold in one minute of synthesis. Further elongation was proportional to the initial burst of initiation. This suggests that in the presence of LEF-5, 10 times as many elongation complexes were formed, and these complexes synthesized RNA at the same rate as those incubated in the absence of LEF-5. The 10-fold stimulation was less than that seen in the absence of heparin, because in our standard assay, initiation can occur during the entire 12-min duration of the assay and the effect is therefore cumulative. This result was confirmed by an additional experiment in which transcription was initiated in the absence of LEF-5, and LEF-5 was not added until after the addition of heparin. In this case, LEF-5 had no effect, consistent with the results of the previous experiment showing that LEF-5 was only active in the formation of initiation complexes. Therefore, we conclude that LEF-5 functions as an initiation factor in this in vitro system.

Although the data presented here indicate that LEF-5 does not function as an elongation factor in vitro, it is premature to conclude at the present time that this hypothesis has been disproved. It is possible that LEF-5 has a transcription elongation function that requires additional factors, which are missing from our assay. In the in vivo assay used by Harwood et al. (10), all 19 essential LEFs were present in addition to all host factors that may be required, while our assay contained only the four RNA polymerase subunits. Alternatively, it is possible that the transcription elongation function of LEF-5 requires a posttranslation modification, like phosphorylation, that does not occur in *E. coli*. In vitro systems with purified proteins are preferred for studies of mechanism, but the disadvantage is that they are limited by the availability of purified proteins. A complete reconstitution of the baculovirus transcription system will be necessary to fully define the functions of all of the LEFs.

LEF-5 increased transcription from two different promoters to an equal extent. The *39k* promoter is a standard late promoter, and the determinants of transcription are limited to the sequences surrounding the consensus TAAG motif. Polyhedrin is a very late promoter whose transcription is regulated by the TAAG motif as well as an A+T-rich sequence downstream of the transcription initiation site. Polyhedrin and *39k* are differentially transcribed in cells (7, 26, 36). Purified baculovirus RNA polymerase specifically recognizes these two promoters but does not discriminate between them, although crude extracts contain a protein that affects promoter choice (8, 35). The fact that LEF-5 affected initiation equally at both promoters suggests that it does not interact with the DNA; rather, it probably increases the ability of the polymerase to form initiation complexes after the polymerase recognizes baculovirus promoters. For example, it is possible that LEF-5 assists in the formation of an open complex or in escape from abortive initiation. Further experimentation will be required to define the mechanism of LEF-5 action and the role of the zinc ribbon in that function.

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