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<td>Jeon, Choon Ju; Yoon, Ho Sup; Agarwal, Kan</td>
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The transcription factor TFIIS zinc ribbon dipeptide Asp-Glu is critical for stimulation of elongation and RNA cleavage by RNA polymerase II

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ABSTRACT

The eukaryotic transcription factor TFIIS enhances elongation and nascent transcript cleavage activities of RNA polymerase II in a stalled elongation complex. By site-directed mutagenesis, we have demonstrated that invariant residues Asp-261 and Glu-262 of the nucleic acid-binding TFIIS Zn ribbon are critical for stimulation of both elongation and RNA cleavage activities of RNA polymerase II. Substitution of either of these residues inactivates both TFIIS functions, suggesting a related role in both activities. These acidic residues may participate in phosphoryl transfer reactions by a two-metal-ion mechanism in a manner analogous to Klenow fragment. The polymerase II itself may contain a Zn ribbon, in as much as the polymerase's 15-kDa subunit contains a sequence that aligns well with the TFIIS Zn ribbon sequence, including a similarly placed pair of acidic residues.

Transcription elongation involves an intricate choreography of protein—protein and protein—nucleic acid interactions, but the details of these interactions are not understood. A conceptual framework for analysis of elongation is represented by the "transcription bubble," which is composed of template and non-template DNA strands, the RNA transcript, RNA polymerase, and associated factors (1). Elongating complexes frequently encounter pause sites along the genes, which are made up of specific sequence elements (2, 3) whose function may be regulated in response to signaling pathways (4). Although the proteins involved in such regulation in vivo are not known, two elongation factors have been identified in the eukaryotic system: TFIIS (also designated S-II; ref. 5) and SIII (6). These proteins are not related and have distinguishable activities. TFIIS stimulates RNA polymerase (pol II) read-through at specific pause or termination sites (7, 8). In addition, it stimulates a pol II transcript shortening activity in a stalled elongation complex (9, 10). An analogous nuclease activity has been shown to be stimulated by bacterial elongation factor Gre A (11).

Abbreviations: pol II, RNA polymerase II; KF, Klenow fragment.
The function of TFIIS is dependent on binding to pol II in a paused elongation complex; otherwise, it is inactive (9). Insight has been obtained into TFIIS function from biochemical studies of mutants, which revealed two functional domains; the C-terminal zinc-bound-domain (residues 231-280) exclusively binds to nucleic acids, whereas the N-terminal domain (residues 1-230) binds to pol II (12). For biological activity, both the C-terminal and the N-terminal domains are required (12). The three-dimensional structure of the nucleic acid-binding domain, as determined by NMR, revealed a structural motif designated as the Zn ribbon, consisting of a three-stranded $\beta$-sheet and the disordered loop (13). To identify the amino acids in the Zn ribbon that stimulate pol II elongation and transcript cleavage, we have examined by mutant analysis the role of the conserved segment RSADE, which is in the disordered loop of the Zn ribbon. The data show that the DE residues play a critical role in stimulation of both pol II elongation and transcript cleavage.

MATERIALS AND METHODS

Construction of TFIIS Mutants. Wild-type TFIIS and TFIIS mutants were expressed in *Escherichia coli* as fusion proteins containing a His$_6$ tag on the N-terminal end. The expression plasmid was constructed by ligation of a 27-nucleotide duplex,

$$\text{CATGGGGCATCATCATCATCATCC}$$ $$\text{GCCGTCAGTAGTAGTAGTAGGGGATC},$$

encoding the nonapeptide MRHHHHHHHP, into the Nco I site of PT/CEBP (14). In this plasmid the upstream Nco I site is inactivated and the newly created downstream site allows subcloning of the cDNA. The Nco I—Kpn I ICEBP cDNA insert was replaced by the TFIIS Nco I—Kpn I cDNA insert (8).

For mutagenesis of the RSADE region, five oligonucleotides were used: G TTCATCAGCAGCAGCTNNNGGTTTGTACCTGTG(R258),GGTTACCATCAGCNNNACTACGGGTTTGTACCTG(A260),TGTCATTGGTTCNNNAGCACTACGGGTTTGTACC(D261), and GTTGTCA-TGGNNNATCAGCAGCAGGGTTTGTAC(E262), where N represents any nucleotide. Mutagenesis of TFIIS using each of these oligonucleotides was performed as described (15). Each mutant was identified by nucleotide sequence analysis.

Isolation of His-Tagged Proteins. The fusion proteins were purified by immobilized Ni$^{2+}$-affinity chromatography as described (16). To remove minor contaminants, the proteins were further purified on a phosphocellulose column. This purification step resulted in >95% homogeneous protein as judged by SDS/PAGE.

In Vitro Transcription. Transcription of dC-tailed DNA by purified calf thymus pol II has been described (12, 17). Equimolar amounts of wild-type and mutant proteins were used in the assay. Protein concentrations were determined by UV absorption.

Preparation of Stalled Ternary Elongation Complexes and Transcript Cleavage Reactions. Artificially stalled ternary complexes were prepared using dC-tailed DNA,
calf thymus pol II, and indicated triphosphates (see Fig. 3A). A typical 25-μl reaction mixture contained 25 mM Hepes (potassium salt) at pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 6.25 mM MgCl₂, 5 mM spermidine, 100 mM potassium glutamate, 10% glycerol, 4 nM dC-tailed DNA, 3.3 units of pol II, 50 μM GTP, and 1 μM[α-³²P]CTP (3000 Ci/mmol; 1 Ci = 37 GBq). This mixture was incubated for 5 min at 30°C. The solution was divided into two aliquots. One of these aliquots was adjusted to 50 μM GTP, 5 μM CTP, and 50 μM UTP (designated +U pause) while the other (designated −U pause) was adjusted with a compensatory volume of buffer. Both reactions were incubated at 30°C for 5 min. Samples were depleted of triphosphates and Mg²⁺ by stepwise change of buffer by using Microcon-10 microconcentrator (Amicon) units at 4°C.

The stalled elongation complexes were analyzed for transcript cleavage. In a typical reaction, 1.5 μl of a stalled complex solution was diluted to 15 μl in the same buffer and incubated at 37°C for the indicated times with or without 6.7 mM MgCl₂, 0.6 μM TFIIS or mutant protein, and 0.35 M KCl. The reactions were stopped by addition of 1 μl of 0.5 M EDTA followed by extraction with phenol/chloroform. The nucleic acids were ethanol precipitated with 2 μg of tRNA and analyzed on 20% polyacrylamide/8 M urea gels.

RESULTS

Rationale for Mutagenesis. To identify the amino acid residues in TFIIS that play a role in stimulation of pol II elongation and RNA cleavage activities, we selected the nucleic acid-binding Zn-ribbon domain for mutagenesis. This domain contains a highly conserved sequence QTRSADEP (residues 256-263) in the disordered loop flanked by an antiparallel β-sheet (Fig. 1; ref. 18). We hypothesized that within this sequence, the residues DE may be critical because similar pairs of acidic residues have been shown to be important for polymerases such as Klenow fragment (KF) and human immunodeficiency virus reverse transcriptase (19, 20). Replacement of residues Q, T, and P by C resulted in retention of both stimulation of elongation and RNA cleavage activities, indicating that these residues were not essential for function (summarized in Fig. 2). The remaining residues RSADE were subjected to saturated mutagenesis to assess the role of these residues in TFIIS function.

Isolation of TFIIS Mutant Proteins. Each of the residues in the RSADE sequence was mutagenized by a saturation mutagenesis procedure employing degenerate oligonucleotides (15). The mutant cDNAs were cloned into a T7-based expression vector downstream of a DNA sequence coding for the MRHHHHHHHP peptide (18). Attachment of the His₆ tag to TFIIS and its mutant forms provided a means for affinity purification (16). The Ni²⁺-based affinity chromatography resulted in highly enriched TFIIS proteins that were then purified to >95% homogeneity by phosphocellulose chromatography (data not shown).

Analysis of Stimulation of pol II Elongation by the Mutant Forms of TFIIS. The mutant proteins were tested for stimulation of pol II elongation activity using dC-tailed DNA containing the gastrin gene terminator. Since TFIIS promotes pol II read-through at
the gastrin gene terminator, use of this template allows measurement of the ability of the mutants to promote read-through (12).

Because of the ease in purification of several mutants, we used His-tagged TFIIS and its mutant forms in these studies. The His-tagged TFIIS and TFIIS stimulated pol II elongation equivalently, indicating that the two proteins are identical by this functional criterion (data not shown).

The analysis of the mutants of R258 indicated a strong preference for basic amino acids. R258K and R258H stimulated pol II read-through at wild-type levels, whereas R258L and R258P showed no stimulation (Fig. 3A, panel 1 and Fig. 3B). R258C showed a 3-fold decrease in activity. The nature of the residue S259 is less restrictive. The hydrophobic amino acid I, neutral amino acid G, and basic amino acid K showed wild-type levels of stimulation, whereas the acidic amino acid E was inactive. Similarly, replacement of residue A260 by hydrophobic amino acids T, P, and L showed wild-type levels of stimulation, whereas acidic amino acid E and C were inactive (Fig. 3A, panel 3 and Fig. 3B). These data suggest that R258, S259, and A260 cannot be anionic.

Interestingly, residues D261 and E262 were highly sensitive to substitution by a large number of residues regardless of their character (Fig. 2). For example, substitution by similar-sized but neutral side chains (residues N and Q, respectively) produced inactive proteins. Replacement of residue D261 by E and residue E262 by D reproducibly resulted in complete inactivation (Fig. 3A, panels 4 and 5 and Fig. 3B). These results suggest that residues D261 and E262 are critical for stimulation of pol II elongation. Moreover, amino acids adjacent to these residues cannot be occupied by acidic residues.

Analysis of TFIIS and Its Mutant Forms for Transcript Cleavage Reaction in Paused Elongation Complexes. In situations where the polymerase becomes trapped at the pause site, the transcription complex moves backwards by hydrolyzing RNA (9-11, 21). TFIIS stimulates this transcript cleavage activity as well. Since residues DE are critical in pol II elongation stimulation, we examined whether these same residues were also critical for the transcript cleavage. Two artificially stalled complexes were prepared by employing dC-tailed DNA, purified pol II and a limited amount and number of nucleotide triphosphates (Fig. 4A).

Transcription of dC-tailed DNA with GTP and CTP should synthesize 8-nucleotide RNA, but RNA synthesis consistently proceeded to the +14 and +15 nucleotide positions (Fig. 4A and Fig. 4C, lane 1; short exposures showed two major bands of similar intensity of 14 and 15 nucleotides). This synthesis of longer RNA may have been due to contamination of GTP and CTP by UTP. These elongation complexes were shown to be stable during salt and triphosphate removal treatments (data not shown). These complexes were designated −U pause (Fig. 4A). Interestingly, addition of 50 μMUTP to −U pause complex advanced the complex to +16 and +17 nucleotides (Fig. 4A and Fig. 4D, lane 1), whereas on the basis of the sequence it should have proceeded to +105 nucleotide positions. A similar complex made up of the 16 and 17 nucleotides transcripts could be directly prepared by using 50 μM GTP, 1 μM CTP, and 50 μM UTP. This elongation complex was designated +U pause complex (Fig. 4A). The −U pause complex could be chased into full-length transcripts in the presence of all four triphosphates at 200
μM, whereas the +U pause complex could not be chased (data not shown). These data indicate that the +U pause site represents an intrinsic arrest site.

pol II required Mg$^{2+}$ for cleavage of RNA in the +U pause complex (Fig. 4B, lane 1 vs. 2). However, the rate of cleavage was exceedingly slow and hence required long reaction times for product detection. This low level of RNA cleavage activity in pol II was not due to TFIIS contamination as evidenced by the fact that no change in the rate of cleavage is observed in the presence of 0.35 M KC1, which is known to completely dissociate TFIIS from pol II (Fig. 4B, lane 2 vs. 3). These data are consistent with the previous observations that, in a stalled elongation complex, pol II can cleave RNA in a Mg$^{2+}$-dependent reaction (21).

The rate of Mg$^{2+}$-dependent RNA cleavage by pol II is increased by 400- to 500-fold in the presence of TFIIS in each of −U pause and +U pause complexes (Fig. 4C, lane 2 and Fig. 4D, lane 2). Interestingly, the nature of the RNA products generated from the −U pause complex was distinct from the +U pause (Fig. 4C, lane 2 vs. Fig. 4D, lane 2). These results are consistent with the previous finding that RNA cleavage pattern in elongation-competent and -incompetent ternary complexes could be different (22).

For analysis of the mutants, two representative mutants for each residue in the SADE sequence were selected. Interestingly, the mutants that were active in stimulation of elongation were also active in RNA cleavage in the −U pause and +U pause complexes. For example, mutants S259I and A260L, which were active in elongation stimulation, were also active in augmentation of RNA cleavage in both stalled complexes (Fig. 4C, lanes 3 and 5 and Fig. 4D, lanes 3 and 5). The remaining mutants S259E, A260E, D261A, D261E, E262A, and E262D were reproducibly inactive in the RNA cleavage reaction in both −U and +U complexes (Fig. 4C, lanes 4 and 6-10 and Fig. 4D, lanes 4 and 6-10); the same mutants were also inactive in elongation stimulation (Fig. 3B). A summary of the mutant data is presented in Fig. 2. These results suggest that residues D and E are indispensable and may play a critical role in both stimulation of elongation and RNA cleavage.

**DISCUSSION**

The frequent regulation of eukaryotic gene expression at a step after initiation of transcription emphasizes the need to understand the biochemical mechanism that controls elongation by pol II. However, there has been little progress in our knowledge of the architecture of the elongation complex, the causes of pausing and termination, and the protein factorsthat regulate the process. In an attempt to delineate such a process, we have undertaken structural and functional studies on the pol II elongation factor TFIIS.

Structural—functional studies on human TFIIS revealed that it is composed of two interacting domains: a N-terminal domain that interacts exclusively with pol II and a C-terminal domain that binds nucleic acids and exhibits a Zn-ribbon structure (12, 13, 18). Since specific mutations in the Zn ribbon did not alter pol II binding, we surmised that this domain may participate in enhancement of both pol II elongation and transcript
cleavage. Thus, mutational analysis of the Zn ribbon ought to shed some light on the
mechanism of action of TFIIS.

Mutant analysis of the Zn ribbon demonstrated that invariant residues D261 and E262 are
critical for stimulation of pol II elongation and transcript cleavage functions in two
distinct stalled elongation complexes. While we had expected to find different amino
cids in the Zn ribbon to be involved in each of pol II functions, we found no mutant with
altered activity in one and not the other function. All of the mutants examined were either
as active as wild-type TFIIS or inactive in stimulating both of the pol II functions. We
therefore conclude that D261 and E262 of TFIIS play critical roles in both pol II
functions.

How does the same dipeptide of TFIIS facilitate both transcript elongation and
transcript cleavage by pol II? Based on the proofreading activity of KF of DNA
polymerase, which is analogous to certain features of pol II and pol II•TFIIS transcript
cleavage, we speculate that the cleavage mechanism of these two enzymes may be similar.
Pausing due to weakness in pairing of the 3´ RNA nucleotide with the DNA may
misalign the 3´-OH in the active site, creating a situation analogous to that of DNA
polymerase-catalyzed misincorporation. In both cases, the 3´ terminal base pair must be
stably paired for resumption of elongation. In KF, 3´→5´ exonuclease allows excision of
the mispaired nucleotide and subsequent incorporation of the correct nucleotide, thereby
creating a stably paired 3´ end; in pol II and pol II•TFIIS, a 3´→5´ RNase activity may
accomplish this task.

Structural and functional studies of KF identified a catalytically critical pair of acidic
residues in the exonuclease site that catalyzes phosphoryltransfer reactions through
coordination of two metal ions (19). Since TFIIS stimulates pol II transcript cleavage in
Mg^{2+}-dependent reaction and requires D261 and E262 for the activity, it is reasonable to
suggest that these acidic residues may participate in RNase activity in an analogous
manner to the KF acidic residues required for exonuclease activity. The mechanism of
pol II and pol II•TFIIS RNase cannot be similar to the RNase A, which generates 3´-
cyclic phosphate and 5´-OH because products of pol II and pol II•TFIIS contain 3´-OH
and 5´-phosphate, which are similar to the products produced by KF exonuclease. We
therefore suggest that D261 and E262 of TFIIS may coordinate Mg^{2+} and participate in
phosphoryltransfer reaction catalyzed by pol II.

The transcript cleavage reaction catalyzed by pol II itself could involve a structural
motif similar to that of Zn ribbon. Indeed, data base searches and structural motif
homology modeling revealed a 15-kDa pol II subunit (Fig. 1). This 15-kDa subunit has
the potential of folding into a Zn-ribbon structure similar to that of TFIIS. While
sequence homology between 15-kDa presumed Zn ribbon and that of TFIIS zinc ribbon
is 40%, the amino acid alignment of the region containing acidic residues is interesting.
The human and Drosophila 15-kDa proteins show a pair of acidic residues at the identical
positions of the TFIIS Zn ribbon, whereas the yeast protein shows only one acidic residue
at these positions. However, the nature of these residues is not identical. Based on the
strict requirement of D261 and E262 for TFIIS function, the 15-kDa subunit Zn ribbon
may not be a functional homolog of TFIIS. In yeast, RPB9 (encoding the yeast 15-kDa
subunit; Fig. 1) is not an essential gene (23), whereas the Drosophila homolog is essential
To shed some light on the role of the 15-kDa subunit in pol II function, it would be interesting to determine whether inactivation of both 15-kDa and TFIIS genes is lethal (in yeast, the gene encoding for TFIIS is also not essential).

In summary, we have shown that residues D261 and E262 in the Zn-ribbon TFIIS are critical for both stimulation of elongation and transcript cleavage by pol II in a stalled transcription complex. A 15-kDa subunit of pol II shows structural homology to TFIIS Zn ribbon and contains acidic residues at the same positions as that of D261 and E262. In analogy to the KF 3´→ 5´ exonuclease, we propose that D261 and E262 in the Zn ribbon TFIIS may participate in two-metal-ion phosphoryltransfer reactions.

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List of Figures

FIG. 1. Amino acid alignment of Zn-ribbon sequences of TFIIS and RNA polymerase II 15-kDa subunit. The C residues, which coordinate zinc, are aligned and shaded. The sequence in the disordered loop is aligned, shaded, and boxed. Residues DE in the box are aligned and boldfaced. β-Sheet residues in THIS and the aligned putative residues in pol II subunit are designated by β at the top.

FIG. 2. Summary of the mutant results. The wild-type sequence is presented in the middle. Because both stimulation of elongation and transcript cleavage activities were coincident for all mutants examined, they have been categorized as active and inactive.

FIG. 3. Analysis of pol II elongation by TFIIS Zn-ribbon mutants. (A) Analysis of the effect of mutant forms of TFIIS on pol II transcription of dC-tailed template. The RNA was synthesized in the presence of [α-32P]CTP. Equimolar amounts of wild-type and mutant proteins were used in the assays. Transcription by pol II alone (lane 1), pol II plus wild-type TFIIS (lane 2), and pol II plus mutants (lanes 3-7 for panels 1-3 and lanes 3-8 for panels 4 and 5) are shown. Positions of full-length (174 nucleotides) and terminated transcripts (96 nucleotides) are indicated. (B) Histogram showing influence of TFIIS and its mutants on pol II transcription activity. The influence of mutant proteins on pol II transcription was measured by comparing total amounts of RNA synthesized in the presence of wild-type and mutant proteins. pol II activity was arbitrarily assigned 100. Quantitation of bands was done by scanning several autoradiographs representing different exposure times on an LKB laser densitometer.

FIG. 4. Enhancement of pol II transcript cleavage of stalled complexes by TFIIS Zn-ribbon mutants. (A) Schematic of the plan to produce −U pause and +U pause complexes. The partial sequence of the dC-tailed DNA is shown. The experimental plan leading to isolation of stalled complexes is given. (B) RNA cleavage by pol II in +U pause complex. RNA cleavage in the +U pause complex (lane 1), the +U pause complex plus Mg2+ (lane 2), and the +U pause complex plus Mg2+ and KCl (lane 3) are shown. Cleavage reactions were done at 37°C for 30 min. The gel was autoradiographed for 24 hr at −70°C. (C) Analysis of the effect of mutant forms of TFIIS on transcript cleavage in the −U pause complex. Transcriptional stimulatory activities of the mutants are indicated at the top. Equimolar amounts of wild-type and mutant proteins were used in the assays. RNA cleavage
reaction in the −U pause complex (lane 1), the −U pause complex plus TFIIS plus Mg$^{2+}$ (lane 2), and −U pause complex plus mutants plus Mg$^{2+}$ (lanes 3-10) are shown. Cleavage reactions were done for 10 min. The gel was autoradiographed for 16 hr at −70°C. (D) Transcript cleavage in the +U pause complex in the presence of mutant forms of TFIIS. Transcript cleavage reaction in the +U pause complex (lane 1), the +U pause complex plus TFIIS and Mg$^{2+}$ (lane 2), and +U pause complex plus mutants and Mg$^{2+}$ (lanes 3-10) is shown. Reactions were for 10 min. Autoradiography was for 8 hr at −70°C.
FIG. 1
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FIG. 2
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