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Stimulation of Transcript Elongation Requires both the Zinc Finger and RNA Polymerase II Binding Domains of Human TFIIS†

Kan Agarwal,* KwangHee Baek, ChoonJu Jeon, Kenichi Miyamoto, Akemichi Ueno, and HoSup Yoon
Departments of Biochemistry and Molecular Biology and Chemistry, The University of Chicago, Chicago, Illinois 60637
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ABSTRACT: The eukaryotic transcriptional factor TFIIS enhances transcript elongation by RNA polymerase II. Here we describe two functional domains in the 280 amino acid human TFIIS protein: residues within positions 100–230 are required for binding to polymerase, and residues 230–280, which form a zinc finger, are required in conjunction with the polymerase binding region for transcriptional stimulation. Interestingly, a mutant TFIIS with only the polymerase binding domain actually inhibits transcription, whereas a mutant in which the polymerase binding and zinc finger domains are separated by an octapeptide is only weakly active. The zinc finger itself has no effect on transcription, but in contrast to the wild-type protein, it binds to oligonucleotides. These findings suggest that TFIIS may interact with RNA polymerase II such that the normally masked zinc finger can specifically contact nucleotides in the transcription elongation zone at a position juxtaposed to the polymerization site.

Regulation of transcript elongation by RNA polymerase II (pol II)[1] is emerging as an important mechanism for gene control in eukaryotic cells. A number of cellular and viral genes (Bentley & Groudine, 1986; Wright & Bishop, 1989; Bender et al., 1987; Reddy & Reddy, 1989; Spencer & Groudine, 1990) are now known to be regulated at the level of transcript elongation. Specific cis-acting transcriptional blocks, located within the transcriptional unit and generated under specified cellular conditions, have been shown to mediate the control of transcript elongation in these examples (Bentley & Groudine, 1986; Reines et al., 1989). That purified pol II can preferentially recognize some of these transcriptional block sites (Diedrick et al., 1987; Kerppola & Kane, 1988) suggests that occasional read-through of such sites in vivo may be mediated by elongation factors (Reines et al., 1989). Involvement of a factor(s) that is capable of influencing elongation is further indicated by the fact that the rate of RNA synthesis by purified pol II is 20–30-fold slower than the in vivo rate of 1000 nucleotides/min. (Ucker & Yamamoto, 1984). One such elongation factor, TFIIS (initially named SII; Natori, 1982), which is capable of stimulating purified pol II transcription, has been identified in mouse (Natori et al., 1973), calf thymus (Rappaport et al., 1987), human (Reinberg & Roeder, 1987), yeast (Sawadogo et al., 1980), and *Drosophila* (Sluder et al., 1989) cell extracts.

Biochemical analysis of TFIIS indicates that it is a phosphoprotein that can stimulate pol II transcription of calf thymus DNA 2–3-fold (Sekimizu et al., 1979) and that of dC-tailed DNA 4–5-fold (SivaRaman et al., 1990). Moreover, purified TFIIS can promote read-through at specific sites within viral and cellular genes whose transcription had been initiated by defined initiation factors (Reines et al., 1989). Thus, TFIIS can stimulate transcription and promote read-through of elongation blocks.

It has been suggested that TFIIS exerts its influence on transcript elongation by binding to pol II without interacting with DNA or NTP (Reinberg & Roeder, 1987; Horikoshi et al., 1984). Further, it has been found that TFIIS binds specifically to the phosphorylated form of the C-terminal domain (CTD) of the largest subunit of pol II a/o (Sawadogo et al., 1980). The potential significance of the interaction between the CTD and TFIIS is indicated by recent data implicating involvement of the CTD in transcriptional elongation (Laybourn & Dahmus, 1989; Sawadogo & Sentenac, 1990; Corden, 1990). While the CTD may be the major contact site for TFIIS, other regions of the subunit may also be involved in TFIIS interactions based on studies with a fusion protein containing a fragment of the largest pol II subunit (Rappaport et al., 1988). Binding of TFIIS to DNA under low salt conditions has been observed previously (Sawadogo et al., 1981), but the physiological significance of this binding is unclear because TFIIS is a basic protein (p*I* 8.7).

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* Author to whom correspondence should be addressed.

[1] Abbreviations: pol II, RNA polymerase II; TFIIS, transcript elongation factor (or SII); CTD, C-terminal domain of the RNA polymerase II largest subunit; cDNA, complementary DNA; Δ, indicates deletion; ∇, indicates insertion; T7, T7 phage RNA polymerase; BSA, bovine serum albumin; EXAFS, extended X-ray absorption fine structure; dsDNA, double-stranded DNA; ssDNA or ssRNA, single-stranded DNA or RNA; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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cDNAs encoding mouse (Hirashima et al., 1988), *Drosophila* (Marshall et al., 1991), and human (Yoo et al., 1991) TFIIS have recently been isolated. It is interesting that the mouse and human proteins are virtually identical in amino acid sequence (Yoo et al., 1991). They share an overall identity of 96%; the similarity increases to 98% if conservative amino acid substitutions are taken into account. Human TFIIS protein (280 residues) is 21 residues shorter than the mouse protein; the gap lies near the N-terminal region. Potential Ser/Thr phosphorylation sites are also localized in the N-terminal 100 residues (Horikoshi et al., 1985). Sequence analysis of the TFIIS protein revealed similarity to the *Drosophila* TFIIS and the yeast PPR2 protein. While the *Drosophila* protein showed 56% identity and 72% similarity to mouse or human proteins, the yeast PPR2 protein exhibited 70% identity to the C-terminal 44 residues of TFIIS (Davies et al., 1990). It is not known whether the PPR2 protein is a candidate for the yeast homologue of TFIIS.

The molecular mechanism by which TFIIS stimulates transcription as well as read-through of elongation blocks is not understood. To elucidate the structural basis of these unique functions and to test the functional significance of some of the TFIIS structural motifs, we have made several mutated forms of TFIIS and assessed their ability to stimulate transcript elongation by pol II. Our results reveal that an internal segment of 131 amino acids (residues 100–230) includes a region required for pol II binding. The carboxyl-terminal domain, which contains a zinc finger, is required in combination with polymerase binding region (residues 100–230) for stimulation of transcription. The zinc finger domain binds to single-stranded (ss) and double-stranded (ds) DNA and RNA and RNA/DNA hybrids. The binding of the TFIIS zinc finger to nucleic acids is discussed in relation to a possible mechanism of TFIIS-mediated stimulation of transcription and read-through of elongation blocks.

Materials and Methods

Construction of TFIIS Mutants. Two previously described plasmids (Yoo et al., 1991) were used in the construction of deletion and insertion mutations. The plasmid pHIIS44 contained the *Eco*RI–*Kpn*I fragment of the human TFIIS cDNA cloned into the *Eco*RI and *Kpn*I sites of pUC18. The *Escherichia coli* expression plasmid pEIIS was derived from the previously described clone pT CEBP (Landschulz et al., 1989). The *Nco*I–*Kpn*I CEBP cDNA insert was removed from pT CEBP and replaced by the TFIIS *Nco*I–*Kpn*I cDNA insert. In this construct, expression of the TFIIS gene is under the direct control of a T7 promoter (Studier & Moffatt, 1986). All DNA manipulations followed general methods previously described (Sambrook et al., 1989). Typically, construction of N-terminal and C-terminal deletions involved replacement of deleted DNA regions by DNA linkers that reintroduced lost met (initiation) and amber (stop) codons, respectively, and simultaneously maintained the original coding frame. The linker sequence was designed to provide a new restriction endonuclease site that served as a convenient marker for ready characterization of the mutant. Similarly, insertion mutants were constructed by introduction of a duplex encoding the desired amino acids, and the ends of the duplex were tailored to maintain the original frame. For the construction of a Δ1–100 deletion mutant, the pEIIS DNA was digested with *Nco*I and *Xho*I, dephosphorylated, purified by gel electrophoresis, and ligated to the phosphorylated DNA linker

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CATGGAAGCTTGCGC
    CTTCGAACGCGAGCT
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This linker encoded the peptide MEAC, in which the ME of TFIIS was retained. Similarly, the mutant Δ1–174 was constructed by replacing the *Nco*I–*Eco*RV region of the TFIIS cDNA by the linker

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CATGGCTCGAAT
    CGAGCTTA
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that introduced a MARI sequence on the N-terminal end. Mutants Δ103–171 and Δ230–280 required replacement of *Xho*I–*Eco*RV and *Bal*I–*Spe*I regions with the DNA linkers

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TCGATCGCGAAT and CCGAGTCATGA
   ACGGCTTA      GGCTCAGTACT
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respectively. No new amino acids were introduced in the Δ103–171, and the dipeptide ES was introduced at the C-terminus of the Δ230–280 protein.

To construct the 230▽231 insertion mutant, we digested pEIIS DNA with *Bal*I, and inserted the oligonucleotide duplex

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CCACTTCGGGCCCAGGTTCAGGGCCC
GGTGAAGCCCGGGTCCAAGTCCCGGG
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that encoded an octapeptide, TSGPGSGP. The unique *Apa*I site present in the DNA linker provided a rapid screening method, but the final characterization of the construction was based upon the nucleotide sequence analysis.

Deletion of residues 165–174 from the TFIIS gene followed the oligonucleotide-directed mutagenesis procedure of Kunkel et al. (1987). For the construction of Δ165–174, the *Eco*RI–*Kpn*I TFIIS cDNA fragment was subcloned into the polylinker of M13mp18. The single-stranded phage DNA was annealed with the mutagenic oligonucleotide TATATAGTTCTTTATTCCTTATGTCTGTACAGTT-TAGAATTTCTACGTTT and extended by T4 DNA polymerase. Identification of the mutant was made by subjecting several randomly selected phage clones to nucleotide sequence analysis. For the construction of the expression plasmid, the *Nco*I–*Kpn*I fragment was isolated from the mutant RF DNA and subcloned into *Nco*I and *Kpn*I digested pT CEBP.

Purification of TFIIS Mutant Proteins. The method described previously for purification of TFIIS from *E. coli* cells harboring a TFIIS expression plasmid (Yoo et al., 1991) is laborious and results in low yields of TFIIS. We describe here a substantially modified purification method that allows rapid isolation of both wild-type and mutant proteins in high yields. Preparation of extract from 10 g of cells follows the previously described procedure (Yoo et al., 1991) except that the protease inhibitors were replaced by TLCK (1 mM), TPCK (1 mM), PMSF (0.2 mM), and benzamidine (5 mM). Removal of nucleic acids from the extract was accomplished by treatment with 0.35% poly(ethyleneimine) instead of 1.2% streptomycin sulfate. Precipitation of proteins including TFIIS by ammonium sulfate at 55% saturation was done as described previously. The ammonium sulfate precipitate was suspended in 60 mL of buffer B (50 mM sodium phosphate, pH 7.4, 100 mM $(NH_4)_2SO_4$, 0.2 mM NaEDTA, 50 mM NaCl, 20 mM 2-mercaptoethanol, 10 μM $ZnSO_4$, 1 mM TPCK, 1 mM TLCK, 5 mM benzamidine, and 0.2 mM PMSF). The solution was then heated (with gentle stirring) in a water bath to 80 °C and kept at this temperature for an additional 5 min before being cooled on ice. After the solution was stirred for 30 min at 4 °C, the precipitated proteins were removed by centrifugation (10000 rpm for 45 min). The clear supernatant was dialyzed against buffer C (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 10% glycerol) for a total of 4 h with three buffer changes. The dialyzate was centrifuged (10000 rpm for 30 min), and the clear supernatant was loaded onto a phosphocellulose (P-11)

column (1 cm × 8 cm) that had been preequilibrated with buffer C. Most of the TFIIS variants bound to the P-11 column with the exception of the mutant Δ165–174 protein. Bound protein was eluted with a linear NaCl gradient of 100 mL (0.05–0.5 mM). The unbound mutant Δ165–174 protein was further purified on an AcA-54 gel filtration column (2.5 cm × 90 cm). While wild-type protein was stable in storage at 4 °C in buffer C plus 300 mM NaCl at protein concentrations greater than 1 mg/mL, the mutant proteins were found to be relatively unstable and required 200 μg/mL of acetylated BSA for their stabilization. By 12% SDS–PAGE and Coomassie Blue staining, the purity of the proteins was assessed as approximately 95%. Protein concentrations were estimated by quantitative amino acid composition analysis.

Preparation of ^{35}S-Labeled Wild-Type and Mutant TFIIS. Cells were grown in an M9 minimal medium to an OD_{600} of 0.5 and then induced by addition of IPTG to 0.3 mM and allowed to grow for an additional 30 min. Rifampicin was then added to 200 μg/mL, and cell growth was allowed to continue for another 90 min. [^{35}S]Met (20 μCi/mL of culture) was added, and the cells were grown for an additional 5 min. Cells were centrifuged for 10 min at 5000 rpm, and the cell pellet was washed once with 25 mM HEPES, pH 7.6, 1 mM EDTA. The pellet was resuspended in 0.1 volume of the original culture, frozen in liquid N_2, and stored at −80 °C. Isolation of the labeled TFIIS and mutant derivatives followed the procedure described above. The purity of the labeled protein was assessed by 12% SDS–PAGE followed by autoradiography.

In Vitro Transcription. Calf thymus RNA polymerase II was purified by the method of Hodo and Blatti (1977); the polymerase had the expected subunit composition and was ~90% pure. TFIIS function was measured by use of both calf thymus DNA and dC-tailed DNA as templates. The assay with calf thymus DNA was carried out in 25 μL of a solution containing 10 mM HEPES, pH 7.9, 3.0 mM $MnCl_2$, 4.0 mM $MgCl_2$, 100 μM ATP, 100 μM GTP, 100 μM CTP, 10 μM UTP, 2 μCi of [5,6-^{3}H]UTP (40 Ci/mmol), 8.25% glycerol, 25 μM EDTA, 125 μM DTT, 50 mM ammonium sulfate, and 20 μg/mL calf thymus DNA (Sigma). Purified RNA polymerase II (1.0 unit; 1 unit = the amount of enzyme required to incorporate 1 pmol of CMP or UMP in a 30-min reaction at 37 °C) alone or in combination with TFIIS proteins (7.0 units; 1 unit = the amount of enzyme required to stimulate 1 unit of pol II to 2 units) was added to the reaction mixture, which was then incubated at 37 °C. After 10 min of incubation, the reaction was stopped by the addition of 5 μL of 150 mM EDTA and 1 mg/mL of calf thymus DNA. Processing of the reaction mixtures followed the procedure previously described (Hodo & Blatti, 1977).

The dC-tailed DNA was prepared as described previously (Yoo et al., 1991). Briefly, an *Ava*II–*Bst*NI fragment of the gastrin gene that contained transcriptional terminator (Sato et al., 1986) was uniquely tailed with dC residues by the procedure described previously (Dedrick & Chamberlin, 1985). A typical transcription reaction that contained dC-tailed DNA (2 nM) in 25 mM KOH–HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 6.25 mM $MgCl_2$, 5 mM spermidine, 150 mM potassium glutamate, 10% glycerol, and either RNA polymerase II (1 unit) alone or RNA polymerase II (1 unit) premixed with TFIIS or its mutant proteins (7.0 units) was preincubated at 30 °C for 10 min. The reaction was initiated by the addition of 500 μM ATP, 500 μM GTP, 500 μM UTP, 50 μM CTP, and 10 μCi of [α-^{32}P]CTP (3000 Ci/mmol) in a final volume of 12.5 μL followed by incubation at 37 °C for 10 min. For experiments involving competition of wild-type TFIIS activity by the mutant proteins, the following modifications were used. The preincubation step involving dC-tailed DNA, pol II, and TFIIS was as described. After 10 min of preincubation at 30 °C, the indicated amounts of mutant proteins were added and the incubation continued for an additional 5 min. The reaction was initiated by the addition of the nucleotide triphosphate mixture as indicated above. The reaction was stopped by the addition of 1 μL of 0.5 M EDTA followed by extraction with phenol/chloroform/isoamyl alcohol. The nucleic acids were ethanol precipitated with 2 μg of tRNA and analyzed on 8% acrylamide/8 M urea gels. For quantitation of bands, the autoradiograms were scanned on an LKB laser densitometer scanner.

Glycerol Gradient Analysis. The interactions of pol II with TFIIS proteins were analyzed on a glycerol gradient, by the procedure of Reinberg and Roeder (1987). RNA polymerase II (4.8 μg) and either radiolabeled TFIIS or mutant TFIIS (102 μg, 62 × 10^6 cpm) were incubated at 4 °C for 10 min followed by further incubation at 37 °C for 2 min. The samples were loaded onto a 11.0-mL linear gradient of 15–35% (v/v) glycerol in a buffer containing 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 10 mM ammonium sulfate, 50 mM KCl, and 3 mM DTT followed by centrifugation at 40000 rpm for 16 h at 4 °C in an SW41 rotor. Fractions containing 330 μL were collected from the bottom of the tubes, aliquots of 20 μL were assayed for RNA polymerase II activity, and 10 μL was counted for radioactivity. For the polymerase activity assay, calf thymus DNA was used as the template and [α-^{32}P]CTP was used as the tracer instead of [^{3}H]UTP. Determination of ^{32}P incorporation into the RNA by GF/C filter binding assay posed problems because of the presence of ^{35}S-labeled proteins. This problem was circumvented by treating the reaction mixture with proteinase K (100 μg/mL) at 50 °C for 30 min prior to precipitation of nucleic acids.

Nucleic Acid Binding Activity. Nucleic acid binding of mutant forms of TFIIS was assayed by use of the gel retardation system described by Jones et al. (1987). The indicated protein samples were incubated for 30 min at room temperature with 2 nM labeled DNA or RNA in 200 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES, pH 7.5, 10% glycerol, 0.05% NP-40, 0.1 mM EDTA, and 100 μg/mL BSA in a total volume of 10 μL. Reactions were analyzed with 6% polyacrylamide gels in a solution of 6.7 mM Tris-HCl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA at 4 °C.

Single-stranded deoxyoligonucleotides were labeled with [γ-^{32}P]ATP (Amersham), and the duplex was labeled with Klenow enzyme and [α-^{32}P]dCTP. RNA was synthesized by T7 RNA polymerase from oligonucleotides (Figure 3A) using [α-^{32}P]CTP, and was gel purified. Both DNA duplex and RNA/DNA hybrids were also gel purified. Single-stranded DNAs and RNAs were heat denatured at 90 °C for 10 min and rapidly chilled on ice just before binding assays were performed.

Results

Construction, Expression, Purification, and Stability of Wild-Type and Mutant Forms of TFIIS Protein. To define human TFIIS structural and functional domains that are necessary for stimulation of pol II transcription, several N-terminal, internal, and C-terminal deletions (as well as an internal insertion) in the protein were generated. The procedures for construction of the mutated genes are described under Materials and Methods. The human TFIIS proteins (wild-type and mutant forms) were overproduced in *E. coli* with use of a T7 expression vector (Studier & Moffatt, 1986).

A high level of expression was observed for each of the TFIIS proteins as judged by the presence of a unique band in the crude extract, in comparison to a control extract prepared from induced cells that contained the expression vector with no insert (Yoo et al., 1991). Most of the TFIIS proteins were purified to apparent homogeneity (Figure 1A) by a newly developed, simple, and highly efficient method outlined under Materials and Methods. This new method exploited the observation that wild-type TFIIS retained full activity after being heated at 80 °C for 10 min. It is unlikely that any of the mutant forms of TFIIS are inactivated by the heat procedure alone since their isolation by an alternative nonheating procedure (Yoo et al., 1991) yielded proteins of identical activities, although the degree of purification was significantly higher with the heating method.

Many of the mutant proteins (Δ103–171, Δ165–174, and 230▽231) were full length at the time of their isolation but later were degraded. This problem was overcome by storing these proteins in the presence of 200 μg/mL BSA at −20 °C. In contrast, the mutant protein Δ174–231 was rapidly degraded in crude extracts even though a full-length form of the protein band was observed in SDS extracts of cells. Attempts to isolate this mutant protein by several modified procedures failed.

Analysis of Transcriptional Activities of the Mutant Forms of TFIIS Proteins. The ability of TFIIS expressed in *E. coli* to stimulate pol II transcription was tested with use of purified calf thymus pol II (Hodo & Blatti, 1977) and calf thymus and dC-tailed DNA templates (Kadesch & Chamberlin, 1982). The transcriptional stimulation with these proteins was 2–3-fold with use of calf thymus DNA (see Figure 5) and 4–5-fold with dC-tailed template (Figures 1C and 3B, panel a), values that are similar to those obtained with purified calf thymus TFIIS (Figure 1C, lanes 1 and 2). Therefore, by these functional criteria, the two TFIIS proteins are identical (Yoo et al., 1991).

Because earlier work suggested that TFIIS augmented transcription exclusively through its binding to pol II, we initially assumed that inactivity of mutant proteins (Figure 5) was due to a defect in polymerase binding. Our data, however, revealed that the mechanism of TFIIS-dependent stimulation involves more than simple binding to pol II. The deletion mutant Δ1–100 was able to stimulate transcriptional elongation of both calf thymus DNA (see Figure 5) and dC-tailed DNA (Figure 1C, lanes 2 and 4) in a manner indistinguishable from that of the wild-type protein. Thus, the first 100 residues are not required for transcriptional stimulation. However, deletion of the 51 C-terminal residues of TFIIS (Δ230–280) resulted in a protein that failed to stimulate transcription. Instead, the Δ230–280 protein, unlike any of the other mutants, actually depressed basal-level transcription by effecting substantial reduction in the levels of both read-through and terminated transcripts (Figure 1C, compare lanes 1, 2, and 6; lane 6 was exposed twice as long as lanes 1 and 2). This inhibitory effect was dependent on the concentration of the mutant protein used; approximately 50% of pol II activity was inhibited by a 25-fold molar excess of the mutant protein over the pol II amount (data extrapolated from Figure 3B, panel a; also see Figure 3A, panel b). At this molar ratio of pol II to mutant protein, the wild-type TFIIS stimulated pol II activity by 5-fold (Figure 3A, panel a; Figure 3B, panel b). This inhibitory effect suggested that the mutant protein has retained its ability to interact with pol II and that the same pol II site may be targeted by both mutant and wild-type proteins. Two observations support this interpretation. (1)

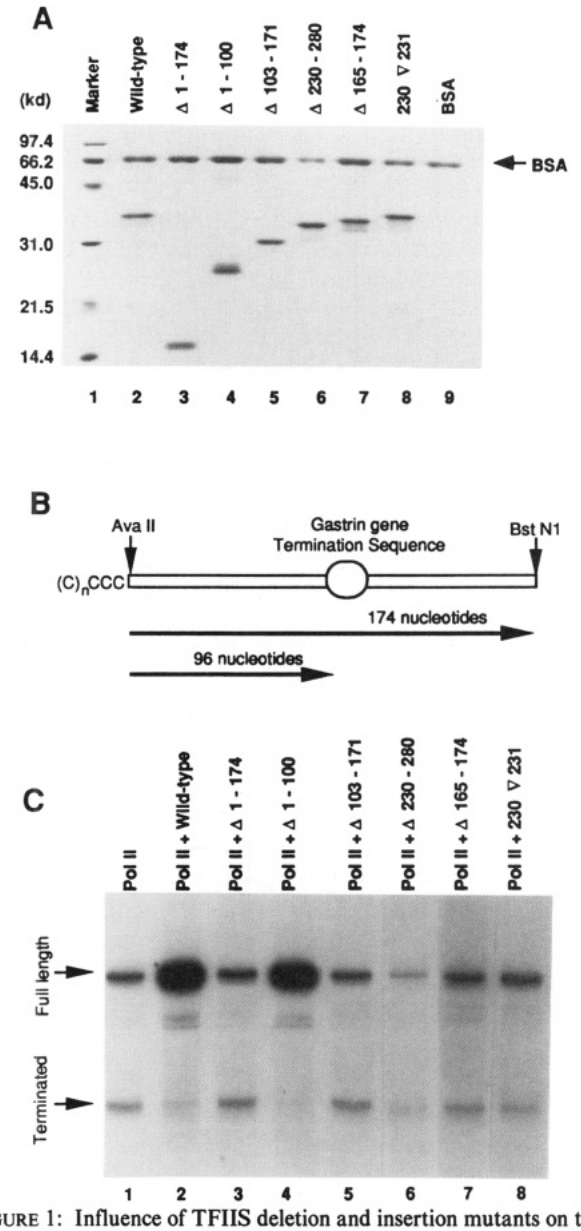


FIGURE 1: Influence of TFIIS deletion and insertion mutants on the transcription of dC-tailed DNA by RNA polymerase II. (A) Analysis of the purified mutant forms of TFIIS by SDS–PAGE. The genes corresponding to the deletion and insertion mutants were expressed in *E. coli*, and the mutant proteins were purified by the newly developed method described under Materials and Methods. The position of the BSA band is indicated; this protein was used to stabilize all of these proteins. The purified wild-type TFIIS (lane 2), the mutant form of TFIIS (lanes 3–7), and the 8 amino acid insertion mutant (lane 8) are shown. Proteins were stained with Coomassie Blue dye. (B) Schematic representation of dC-tailed DNA. The *Ava*II–*Bst*NI fragment of the gastrin gene 3′-flanking region (Sato et al., 1986) was tailed with an average of 40–60 dC-residues on the *Ava*II side of the fragment as indicated. The circle indicates the relative position of the termination site. Transcription of the dC-tailed DNA was carried out with use of purified calf thymus RNA polymerase II as described under Materials and Methods. The sizes of the full-length and terminated RNA products are indicated. (C) Analysis of the effect of mutant forms of TFIIS on transcription of dC-tailed template. The influence of mutant proteins on transcription of RNA polymerase II was measured by comparing total amounts of RNA synthesized in the presence of wild-type and mutant forms of TFIIS. RNA was synthesized in the presence of [α-^{32}P]CTP as described under Materials and Methods. Equimolar amounts of wild-type and mutant proteins were used in the assays. The positions of the full-length and terminated RNAs are indicated. Transcription by RNA polymerase II alone (lane 1), RNA polymerase II plus wild-type TFIIS (lane 2), and RNA polymerase II plus mutants (lanes 3–8) are shown. Samples in lanes 1–5 were autoradiographed for 8 h and in lanes 6–8 for 17 h at −70 °C.

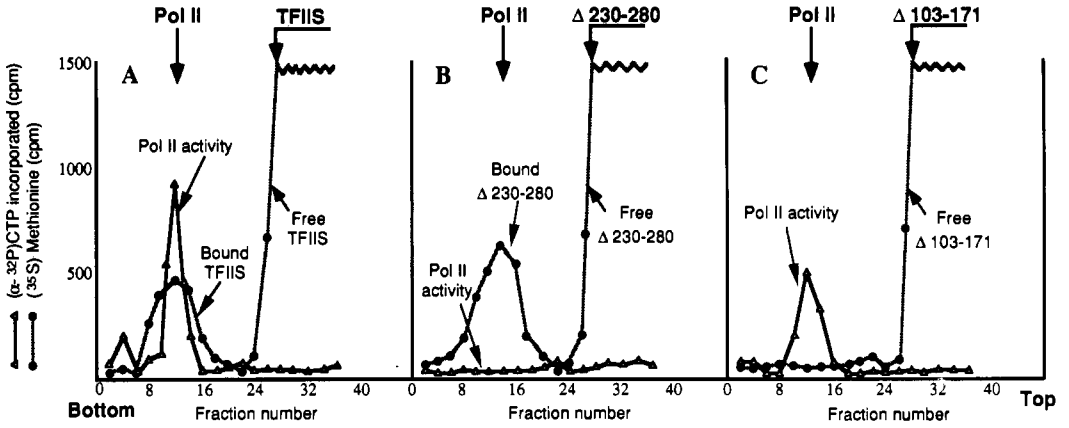


FIGURE 2: Analysis of the interaction of wild-type and mutant forms of TFIIS with purified RNA polymerase II by glycerol gradient sedimentation. Purified polymerase II (4.8 μg, 106 300 units/mg of protein) and purified wild-type or mutant forms of [^{35}S]Met-labeled TFIIS (102 μg, 6 × 10^5 cpm/μg) were subjected to glycerol gradient centrifugation as described under Materials and Methods. The sedimentation positions of RNA polymerase II, wild-type, and mutant proteins are indicated by arrows at the top of each panel. The positions of bound and free forms of TFIIS were determined by counting radioactivity and the positions of RNA polymerase II by assaying its activity. Panel A, pol II + wild-type TFIIS; panel B, pol II + mutant Δ230–280; and panel C, pol II + mutant Δ103–171.

The isolation of a complex of the mutant protein and purified pol II by glycerol gradient centrifugation has a sedimentation value similar to that of the wild-type TFIIS·pol II complex (Figure 2, panels A and B). In these gradients, mutant and wild-type TFIIS sedimented relatively slower than the complexes and the free pol II. Interestingly, the isolated Δ230–280·pol II complex was inactive, while the wild-type TFIIS·pol II complex was active (Figure 2, panels A and panel B); these results are consistent with the studies that involved complexes formed in situ (Figures 1C, lanes 1, 2, and 6). (2) The inhibition of wild-type TFIIS mediated transcriptional stimulation of pol II by Δ230–280 protein in a concentration-dependent manner also supported the interpretation presented above. As shown in Figure 3A, panel d, and Figure 3B, panel b, as much as 60% of the stimulatory activity of wild-type protein could be inhibited by a 5-fold excess of the mutant protein. From these data, we conclude that residues between positions 100 and 230 of TFIIS are important for binding to pol II but that pol II binding by TFIIS is not sufficient for transcriptional enhancement.

In an attempt to localize the polymerase binding region within residues 100–230 further, several deletions that overlapped in portions of this region were analyzed. The mutants Δ1–174 (Figure 1C, lane 3) and Δ103–171 (lane 5) had lost their ability to stimulate transcription; the Δ174–231 protein was extremely unstable and could not be analyzed. Even a deletion as short as 10 amino acids (Δ165–174, a region of alternating basic residues; see Figure 4) inactivated the protein (lane 7). That the inactivity of these TFIIS mutant proteins may be due to a defect in pol II binding was indicated by their inability to influence the stimulatory activity of wild-type TFIIS in competition assays involving transcription of calf thymus DNA (data summarized in Figure 5).[2] Additional evidence for this interpretation emerged from glycerol gradient analysis of binding of the mutant Δ103–171 to pol II. As shown in Figure 2, the inactive Δ103–171 protein showed no detectable binding to pol II in contrast to the active wild-type TFIIS (Figure 2, panel A vs panel C). Together with the data presented above, these results suggest that the pol II binding domain of TFIIS resides in the internal portion of the molecule and that information within residues 165–174 is critical for binding.

The lack of activity of the Δ230–280 mutant to stimulate transcription, despite binding efficiently with pol II, indicated involvement of the C-terminal region in the stimulation of transcription. Contrary to the report that mouse TFIIS does not contain a zinc finger (Hirashima et al., 1988), we have found that residues 230–280 in human TFIIS, which are identical with the same region of mouse TFIIS, contain a single zinc finger, whose structure is shown in Figure 5. Our evidence is based on (1) the estimation of zinc content by atomic absorption spectroscopy,[3] (2) EXAFS analysis,[3] and (3) analysis of site-specific mutants involving the implicated Cys residues.[4] To test whether the zinc finger and pol II binding domains must maintain a precise relative spatial arrangement, a mutant, 230▽231, was made in which these two domains of TFIIS were separated by the insertion of an octapeptide (TSGPGSGP) that is expected to form a random coil. Remarkably, this mutant, in contrast to the inhibition of pol II seen with mutant Δ230–280, displayed only a residual pol II stimulatory activity[5] (Figure 1C, lane 8; Figure 3A, panel c; Figure 3B, panel a), suggesting the possibility that the mutant protein binds to the polymerase but is unable to stimulate transcription. The binding of the mutant 230▽231 protein with polymerase is indicated further by its ability to depress transcriptional stimulation by the wild-type TFIIS (Figure 3A, panel e; Figure 3B, panel b). The fact that the mutant 230▽321 can reduce the activity of the wild-type TFIIS·pol II complex, albeit with reduced efficiency in comparison to mutant Δ230–280, suggests that this mutant can bind to pol II but cannot stimulate or inhibit pol II activity. It is also possible that inhibition of the TFIIS·pol II complex activity is due, in part, to heterodimer

[2] Alternatively, the lack of pol II and nucleic acids binding activities of the Δ165–174 protein may be due to misfolding of the mutant protein.

[3] The zinc content in wild-type TFIIS and mutant Δ1–174 was estimated to be 0.86 and 1.02 mol of zinc atom/mol of protein, respectively (Jeon et al., manuscript in preparation). EXAFS measurements slowed the presence of tetracoordinated zinc (Korszun, unpublished results).

[4] Replacement of any one of the four Cys residues (Figure 5) resulted in the isolation of a truncated protein whose molecular size and biological activities were similar to those of the mutant Δ230–280 protein. This indicates that disruption of the zinc coordination site results in cleavage by an *E. coli* protease at a site located upstream of the zinc finger region. Repeated attempts to isolate intact protein in which any of these four Cys residues was replaced by a Ser were unsuccessful (Jeon et al., manuscript in preparation).

[5] An approximately 20% increase in overall transcription was observed at an 80-fold molar excess of the mutant protein relative to pol II (Figure 3B, panel a). This increase, although small, does indicate interaction of mutant protein with pol II.

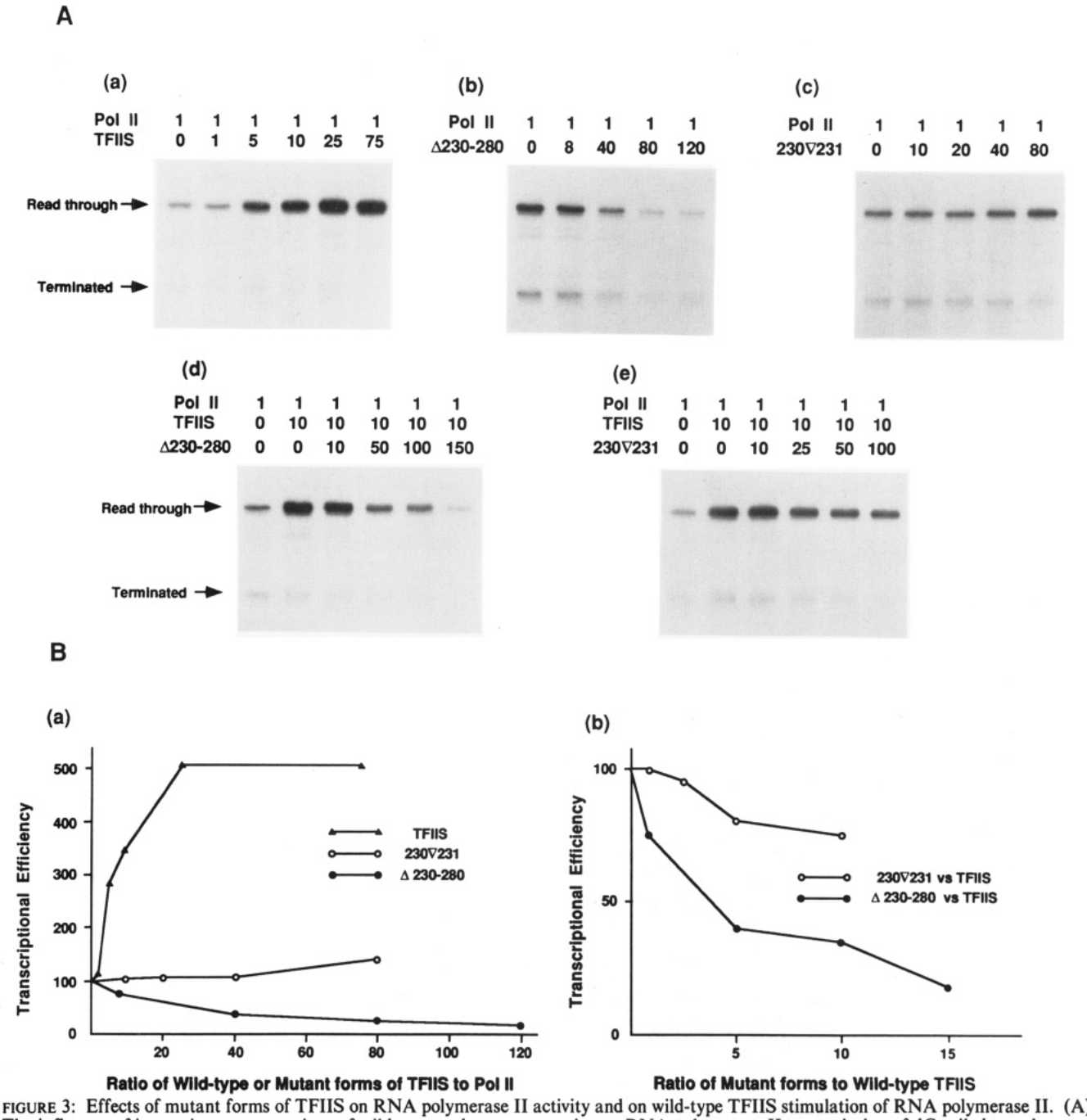


FIGURE 3: Effects of mutant forms of TFIIS on RNA polymerase II activity and on wild-type TFIIS stimulation of RNA polymerase II. (A) The influence of increasing concentrations of wild-type and mutant proteins on RNA polymerase II transcription of dC-tailed template was measured. RNA was synthesized in the presence of [α-^{32}P]CTP, as described under Materials and Methods. The ratios of pol II and TFIIS proteins are indicated at the top of each of the autoradiographs. Panels a, b, and c show the effects of increasing concentrations of wild-type TFIIS, mutant Δ230–280, and mutant 230∇231, respectively, on the read-through and terminated transcripts. With wild-type TFIIS, increased read-through and decreased termination are observed with increasing amounts of TFIIS (panel a). The inhibitory effect of mutant Δ230–280, in panel b, is characterized by a gradual decrease in overall transcription with increasing amounts of the mutant protein. The weak stimulatory effect by the mutant 230∇231 on transcription is shown in panel c. The influence on the transcriptional stimulatory activity of wild-type TFIIS by the mutant Δ230–280 and 230∇231 proteins is shown in panels d and e, respectively. (B) Quantitation of the effect of wild-type TFIIS and mutant proteins on transcription levels. Panel a shows the effect on RNA polymerase II activity, which is arbitrarily assumed at 100%. For the quantitation of bands, several autoradiograms, representing different exposure times, were scanned on an LKB laser densitometer scanner. Panel b represents the data from panels d and e.

formation between the wild-type TFIIS and the mutant proteins, and such dimers could reduce the homodimer concentration required for full activity. This possibility remains untested. Nevertheless, since both functional domains are retained intact in the 230∇231 mutant, these results suggest that both of these domains are required for TFIIS function and that the two could have a precise spatial arrangement.

Analysis of DNA Binding Activities of Mutant Forms of TFIIS. The inability of either of the individual domains of TFIIS alone to stimulate transcription and the failure of the zinc finger domain to bind to polymerase led us to the hypothesis that the zinc finger may stimulate transcription and read-through of elongation blocks by interacting with nonspecific nucleic acids within the zone of transcription. To test this possibility, interactions of wild-type and several selected mutant proteins with dsDNA, ssDNA, RNA, and RNA/DNA hybrid oligonucleotides were examined by gel mobility shift assays.[6] The random oligonucleotides chosen are

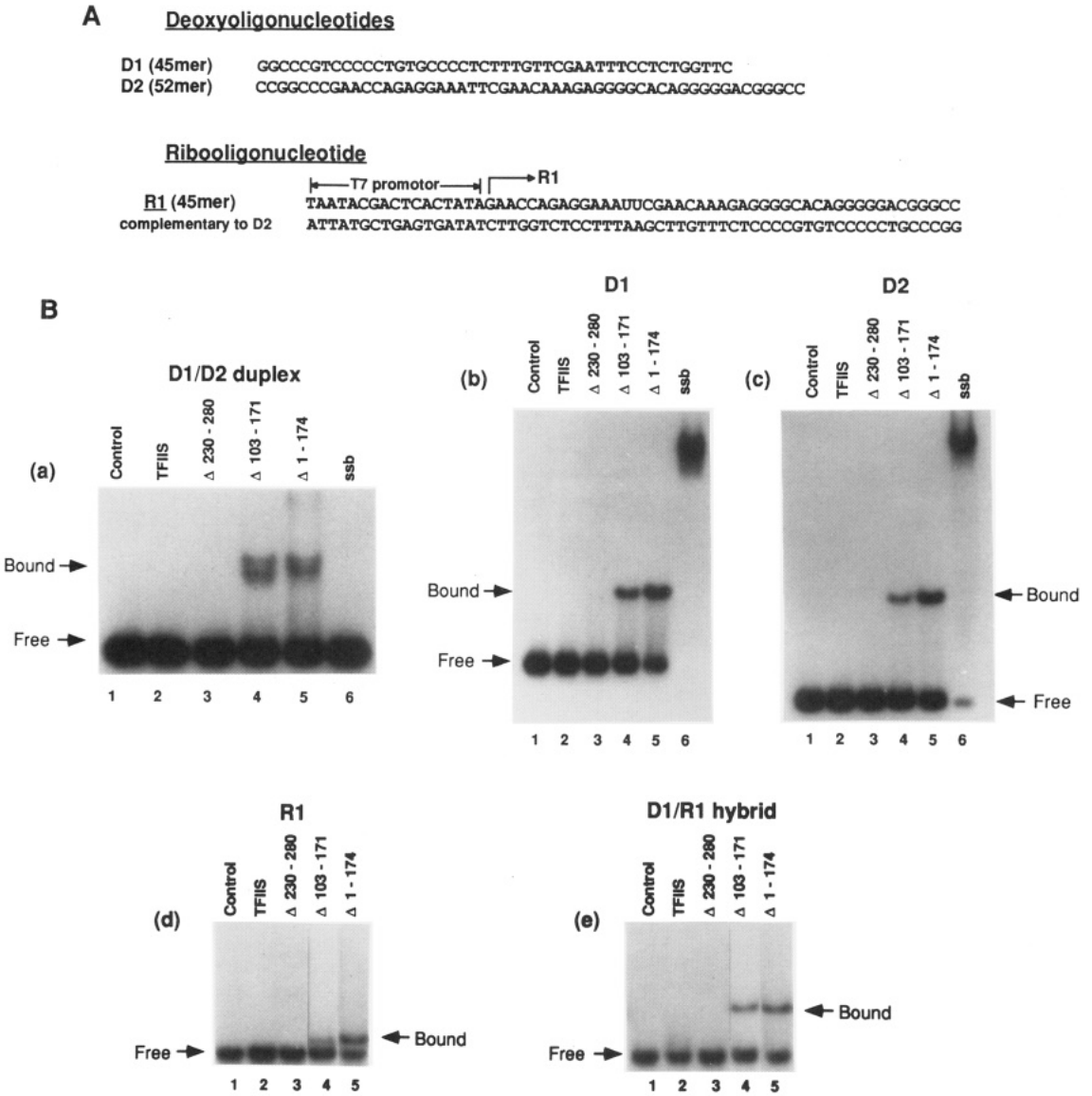


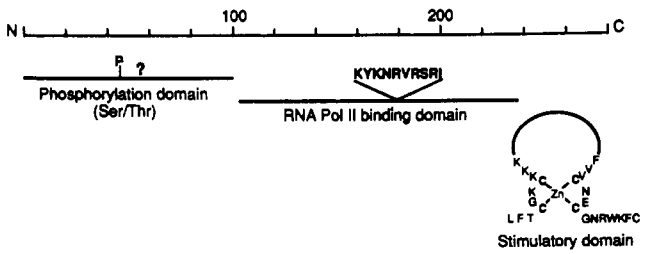
FIGURE 4: Nucleic acid binding activities of the wild-type and mutant forms of TFIIS. (A) Oligonucleotide sequences used in the binding assay are shown. Single-stranded deoxyoligonucleotides (D1 and D2) were labeled with T4 polynucleotide kinase, and the D1/D2 duplex was labeled with the Klenow fragment of *E. coli* DNA polymerase. Ribooligonucleotide R1 was synthesized from the T7 promoter–template hybrid by the T7 RNA polymerase in the presence of [α-^{32}P]CTP as illustrated. Both DNA duplex and RNA/DNA hybrid were gel purified before use. (B) Analysis of nucleic acid binding activity by gel mobility shift assays. In all assays the protein concentrations of wild-type, Δ230–280, and Δ103–171 were maintained at 800 nM and that of Δ1–174 was maintained at 200 nM. The nucleic acid concentration used as 2 nM with the exception of that of the D1/D2 duplex, which was maintained at 4 nM. Binding of all forms of TFIIS to the D1/D2 duplex (1.9×10^6 cpm/μmol, panel a), to D1 (1.6×10^5 cpm/μmol, panel b), to D2 (2.1×10^5 cpm/μmol, panel c), to R1 (2.9×10^6 cpm/μmol, panel d) and to the D1/R1 hybrid (2.9×10^6 cpm/μmol, panel e) is shown. T4 bacteriophage gene 32 protein (ssb) was used as a control and is shown in lane 6. The positions of free nucleic acid and bound protein–nucleic acid complexes are indicated.

presented in Figure 4A. The wild-type TFIIS and mutant Δ1–100 proteins, both of which stimulated pol II activity, failed to bind to any of the oligonucleotides (Figure 4B). The mutant Δ230–280, which depressed pol II function, did not bind to any of these oligonucleotides (lane 3 in panels a–e) either. However, the mutants Δ103–171 and Δ1–174, which are defective in polymerase binding but contain an intact zinc finger domain, showed binding to both duplex (panel a, lanes 4 and 5) and single-stranded DNA (lanes 4 and 5 in panels b and c) as well as to the RNA (panel d, lanes 4 and 5) and RNA/DNA hybrid (panel e, lanes 4 and 5). We conclude that the nucleic acid binding activity resides in the zinc finger domain of TFIIS but that in the isolated wild-type TFIIS protein this region is masked by the pol II binding domain.

The binding affinity of mutants Δ103–171 and Δ1–174 to single-stranded DNA was approximately 5–10-fold higher than to the double-stranded DNA, RNA, and RNA/DNA hybrids. Although the intensities of the bound bands (Figure 4B) appear similar, their estimated specific activities varied 5–10-fold. For instance, the single-stranded oligonucleotides used were 5′-end-labeled and had approximately 6-fold lower specific activity than the duplex DNA, RNA, and RNA/DNA hybrids that contained multiple internally labeled sites.

On the basis of the molecular size difference between the two mutant proteins (approximately 16 kDa for Δ1–174 and 33 kDa for Δ103–171), significantly different mobility shifts would have been expected rather than the very similar shifts actually observed (Figure 4B, lanes 4 and 5 in panels a, b, and

[6] If binding assays are performed in 50 mM KCl according to the procedures reported, a substantial portion of the oligonucleotide precipitates out of the solution, presumably as a protein–oligonucleotide ionic complex. This problem was circumvented, however, when 200 mM KCl was used in the binding assays.



Mutants	Pol II Binding	Transcriptional Stimulation	Transcriptional Inhibition	ss DNA Binding
Wild-type	+[a]	++++ (++)	- (-)	-
Δ 1 - 100	+[b]	++++ (++)	- (-)	-
Δ 1 - 174	-[b]	- (-)	- (-)	+
Δ 103 - 171	-[a]	- (-)	- (-)	+
Δ 174 - 231	ND	ND	ND	ND
Δ 230 - 280	+[a]	- (-)	++ (+)	-
Δ 165 - 174	-[c]	- (-)	- (-)	-
230 ▽ 231	+[b]	- (-)	- (-)	ND

FIGURE 5: Schematic of proposed structural and functional domains of TFIIS and a summary of activities of wild-type and mutant proteins. The sequence of the human TFIIS (Yoo et al., 1991) is depicted at the top of the figure, where the amino and carboxyl termini are marked as N and C, respectively. The Ser/Thr phosphorylation, RNA polymerase II binding, and transcription stimulatory domains are illustrated schematically. The positions of Ser and Thr phosphorylation are unknown (indicated by a question mark). The sequence of the alternating basic residues in the polymerase binding domain is shown, as is the partial sequence of the zinc finger. In the column on the left, the deleted regions (Δ) and the site of insertion (▽) are indicated by amino acid numbers. The level of transcription of dC-tailed DNA using wild-type TFIIS is arbitrarily set at 100% and is indicated by ++++ and that of calf thymus DNA by (++). The minus sign indicates no effect on transcription. The binding of proteins to pol II was determined by (a) glycerol gradient centrifugation or (b) competition with wild-type protein assay, or (c) was inferred from inactivity. ND, not determined.

c). It is very unlikely that the observed DNA binding was due to an *E. coli* protein that copurified with these but not with the other highly purified mutant proteins (Figure 1A) because (1) antibodies prepared against the mutant Δ1–174 protein prevented binding of mutant proteins to single- and double-stranded DNAs[7] and (2) binding affinity of such a protein would have to be extremely high, given the very low levels of non-TFIIS protein present (Figure 1A). One possible explanation of the similar mobility shifts is that the Δ103–171 bound to the DNA was monomer, whereas the Δ1–174 bound to DNA was in a dimerized form. Considering the size of the mobility shift generated by the *E. coli* single-stranded DNA binding protein (ssb, 32 kDa), which, in contrast to the mutant Δ103–171 (33 kDa), is known to bind cooperatively, it is reasonable to assume that it was the monomeric form of the mutant TFIIS that bound to DNA.

Discussion

The frequent regulation of gene transcription at a step after initiation emphasizes the need to understand the biochemical mechanisms that control elongation by RNA polymerase II. The transcription stimulatory factor TFIIS, also known as elongation factor, dramatically influences the intrinsic elongation function of pol II (Reinberg & Roeder, 1987). In this report, we have defined two functional domains of TFIIS in a first step toward understanding the chemical details of TFIIS function. Our results, summarized in Figure 5, reveal that the internal portion of TFIIS binds to RNA polymerase II and that the C-terminal zinc finger can bind to nucleic acids and is needed together with the polymerase binding domain to stimulate transcription. Moreover, the precise relative positioning of these two domains appears to be critical for transcriptional stimulation.

An Internal Segment of TFIIS Is Required for RNA Polymerase II Binding. We have shown that the 180-residue C-terminal portion of human TFIIS (residues 101–280) is sufficient for both biochemical activities that are characteristic of TFIIS, namely, binding to pol II and ability to stimulate transcription. Similar results with a C-terminal 21-kDa proteolytic fragment of mouse TFIIS (corresponding roughly to residues 101–280 of human TFIIS) were reported by Hirashima et al. (1988). Within this 180-residue C-terminal region, the polymerase binding region is located between residues 100 and 230. Interestingly, the Δ230–280 TFIIS variant, which binds to pol II with an affinity similar to that of wild-type protein, actually causes a dramatic reduction in overall transcription level. It is important to note that the wild-type and Δ230–280 mutant utilize similar or identical pol II binding sites since they compete with each other for binding to pol II. Because Δ230–280 possesses pol II binding ability but lacks stimulatory activity, the stimulatory activity must require the deleted C-terminal 51 residues of TFIIS. The dependence of the C-terminal region on an intact pol II binding site for stimulatory function indicates that these two functional domains in TFIIS must operate in concert to augment transcriptional elongation. The finding that insertion of only a small stretch of random coil abolished activity suggests that their interdependent function requires a precise relative structural arrangement.

On the basis of the previous observation that the yeast p37 protein (a functional homologue of mammalian TFIIS) preferentially binds to pol II a/o, a form in which the CTD is phosphorylated (Sawadogo et al., 1980), we postulate that the alternating basic residues between positions 165 and 174 in TFIIS interact with the phosphorylated CTD. In support of this idea, we found that the TFIIS mutant Δ165–174, which is missing this segment, failed to bind to pol II a/o (inferred from its inability to either stimulate or inhibit basal-level transcription and depress stimulatory activity of TFIIS). However, these observations do not rule out interactions of the basic residues with other regions of the pol II largest subunit.

The C-Terminal Zinc Finger Binds to Nucleic Acids. It is striking that whereas intact TFIIS does not bind to nonspecific single- and double-stranded DNAs, RNAs, and RNA/DNA hybrids, removal of portions of the polymerase binding domain results in mutant forms of TFIIS that can in fact bind to these same nucleic acids. This binding activity resides at least in part in the zinc finger since TFIIS mutants lacking the zinc finger failed to bind nucleic acids. These data suggest that there are significant interdomain interactions in the wild-type TFIIS and that such interactions may prevent binding of zinc finger to nucleic acids. Disruption of these interdomain interactions can free the zinc finger and enable it to interact with DNA and/or RNA. We suggest that in

[7] Antibodies prepared against wild-type protein reacted poorly with the zinc finger region, and hence, antibodies raised against the mutant Δ1–174 protein were used in the assay.

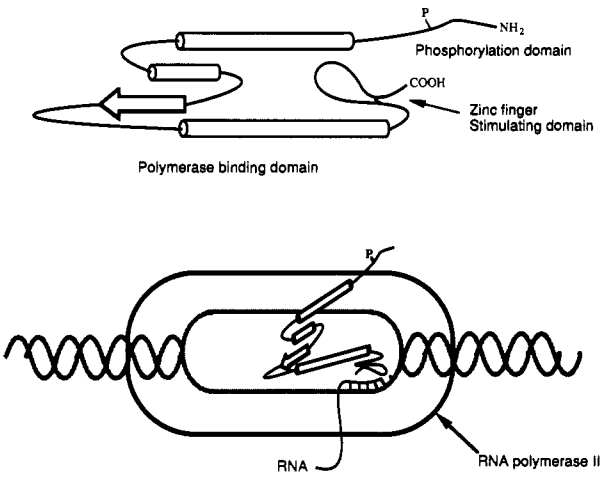


FIGURE 6: A model for transcript elongation. (Top) Predicted secondary structure of the polymerase binding and stimulatory zinc finger domains (Garnier et al., 1978). Interaction of the zinc finger with two surface α-helices as shown is arbitrary. (Bottom) An amplified view of the elongation process showing binding of the internal region (residues 100–230) to polymerase and interaction of the zinc finger with 3′-termini of the RNA/DNA hybrid near the catalytic site of the polymerase.

vivo the masked zinc finger in TFIIS would be freed as a consequence of a conformational change resulting from binding to pol II. Masking the zinc finger through interdomain interactions might be important because it would ensure that TFIIS binding to nucleic acids occurs only if it is bound to pol II.

How Might TFIIS Function. It is tempting to speculate that the transcriptional stimulation, antipausing, and antitermination activities of TFIIS are augmented via its interaction with pol II in the transcription elongation bubble (Figure 6) such that the zinc finger can interact with the appropriate nucleic acid. The observed binding of the zinc finger to ssDNA, RNA, dsDNA, and RNA/DNA hybrids suggests that the zinc finger could potentially act on all four forms in the elongation bubble. All four of these nucleic acid structures would be at or near the bubble in close proximity to the catalytic zone.

The binding of the zinc finger to dsDNA does not appear to be functionally significant because the zinc finger neither promotes unwinding of dsDNA, ATP dependent or independent, nor influences stability of the dsDNA. If binding of the zinc finger to dsDNA was to be functionally important, it would be expected to exhibit dsDNA unwinding activity.[8]

The higher affinity of the zinc finger for ssDNA than for dsDNA, RNA, or RNA/DNA hybrids points to ssDNA binding as the most likely to be functionally relevant. The ssDNA sites that can be considered functionally important in the bubble (Figure 6) are the nontranscribed strand and the first unpaired nucleotide (Shi et al., 1988; Yager & von Hippel, 1991). While it is possible that interaction with the nontranscribed strand could stimulate pol II activity by transient stabilization of the ssDNA region, such an activity would not adequately explain TFIIS's antipausing and antitermination activities or the influence of TFIIS on the optimal concentrations of nucleotide triphosphates required for efficient synthesis of RNA. Interaction of the zinc finger with the first unpaired nucleotide and the 3′-terminal region of the RNA/DNA hybrid in the transcriptional zone (see below) could explain these activities.

The pyrimidine-rich sequences that frequently serve as pausing (Kerppola et al., 1988; SivaRaman et al., 1990) or termination (Sato et al., 1986) sites are specifically rich in thymidine residues and are localized in the nontranscribed strand. Transcription of an adenosine-rich region generates an RNA/DNA hybrid in which the 3′ end of the RNA involves several rU/dA base-pairs. On the basis of the relative weakness of rU/dA base-pairs, particularly when they are segregated near the 3′-terminal region of the RNA, it has been suggested that such weakly paired 3′ ends of the RNA with the DNA template promote pausing and termination of transcription (Yager & von Hippel, 1991). Stabilization of weakly paired 3′-terminal nucleotides of the RNA in the catalytic zone by the zinc finger of TFIIS should, therefore, reduce pausing and termination. Preliminary studies indicate that the zinc finger region is able to bind to an RNA/DNA hybrid that corresponds to the gastrin gene termination sequence, whereas it fails to bind to corresponding RNA or DNA.[9] From these results we suggest that the zinc finger interacts with the 3′ end of the RNA/DNA hybrid in the catalytic zone to prevent transcription pausing and termination.

Since the first unpaired template nucleotide in the bubble is in close proximity to the 3′ end of the RNA/DNA hybrid, the zinc finger region may also influence the interaction of the incoming nucleotide triphosphate with this unpaired template nucleotide (Figure 6). Support for this idea comes from the fact that, in the presence of TFIIS, low NTP concentrations are sufficient (50–100 μM) to bring about efficient RNA synthesis without detectable pausing, while higher concentrations of NTP (500 μM–1 mM) are needed when TFIIS is absent.[10] This result also supports the idea that the zinc finger may also stabilize the 3′-terminal region of the RNA/DNA hybrid because stable 3′ termini of such hybrids would require relatively lower concentrations of the nucleotide triphosphates for efficient elongation.

The identification of polymerase binding and stimulatory zinc finger domains in TFIIS, and the demonstration of the nucleic acid binding ability of the zinc finger, provides a foundation for an understanding of the mechanism by which TFIIS regulates pol II transcription. Our model for TFIIS action, illustrated in Figure 6, can be tested biochemically and genetically. This study should be useful for understanding the regulation of RNA elongation and for the elucidation of the mechanism of transcription antipausing and termination.

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[8] Stability of the preformed duplexed oligonucleotides is not influenced by the mutant Δ1–174 protein in the presence or absence of ATP as judged by gel electrophoresis (Yoon et al., unpublished results).

[9] Yoon et al., unpublished results.

[10] Ueno et al., unpublished results.

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