Yeast Transcript Elongation Factor (TFIIS), Structure and Function
II: RNA POLYMERASE BINDING, TRANSCRIPT CLEAVAGE, AND READ-THROUGH*

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The transcriptionally active fragment of the yeast RNA polymerase II transcription elongation factor, TFIIS, comprises a three-helix bundle and a zinc ribbon motif joined by a linker region. We have probed the function of this fragment of TFIIS using structure-guided mutagenesis. The helix bundle domain binds RNA polymerase II with the same affinity as does the full-length TFIIS, and this interaction is mediated by a basic patch on the outer face of the third helix. TFIIS mutants that were unable to bind RNA polymerase II were inactive for transcription activity, confirming the central role of polymerase binding in the TFIIS mechanism of action. The linker and zinc ribbon regions play roles in promoting cleavage of the nascent transcript and read-through past the block to elongation. Mutation of three aromatic residues in the zinc ribbon domain (Phe269, Phe296, and Phe308) impaired both transcript cleavage and read-through. Mutations introduced in the linker region between residues 240 and 245 and between 250 and 255 also severely impaired both transcript cleavage and read-through activities. Our analysis suggests that the linker region of TFIIS probably adopts a critical structure in the context of the elongation complex.

Elongating RNA polymerase II stalls upon encountering blocks to elongation in vitro (1). In some cases, these transiently stalled polymerases convert to very stable arrested complexes. Arrested complexes are unable to resume transcription even after hours to weeks of incubation (2). The inability of such complexes to resume transcription results from a structural change in the stalled polymerase, which causes the active site to disengage from the 3'-end of the transcript (3). The general elongation factor TFIIS1 reactivates arrested transcription complexes within minutes (4). The reactivation process involves a TFIIS-stimulated endonucleolytic cleavage of the transcript by the RNA polymerase II (5, 6), which relocates the polymerase active site to the new 3'-end of the RNA chain and allows for chain extension.

The reactivation of stalled elongation complexes involves multiple steps, with the first being the interaction of TFIIS with RNA polymerase II. The TFIIS-binding domain on RNA polymerase II was identified by Friesen and colleagues (7), who discovered mutants in the largest subunit of RNA polymerase II, RPB1, that displayed the same phenotype as a strain deleted for the TFIIS gene (sensitivity to the drug 6-azauracil) and that also could be suppressed by overexpression of TFIIS. These mutants localized to a part of RPB1 between regions G and H, which are conserved from bacteria to man and are in close proximity to the RNA polymerase active site (8, 9). The genetic evidence for a TFIIS interacting domain was confirmed biochemically, when two of the mutant RNA polymerases were purified and shown directly to have 500-fold lower affinity for TFIIS compared with the wild-type polymerase (10).

Transcript cleavage is the next essential step in the reactivation process. It is now clear that RNA polymerase II itself is the endonuclease; the enzyme can catalyze the cleavage reaction at elevated pH in the absence of an auxiliary factor (11, 12).2 The role of transcription elongation factors, such as TFIIS, is to dramatically increase the rate of transcript cleavage at physiological pH. Cleavage is thought to be essential for the reactivation process; mutants in TFIIS that are unable to stimulate transcript cleavage cannot stimulate read-through past blocks to elongation. However, cleavage is insufficient to promote read-through (13). After the cleavage event, a conformational change in the arrested complex is possibly required to allow the RNA polymerase to resume transcription from the newly created 3'-end of the transcript (12). The mechanism of the presumed conformational change is the least well understood part of the TFIIS reaction. It is clear that TFIIS is required for the change, but also that other components are involved. For example, RNA polymerase mutants lacking the RPB9 subunit are able to catalyze an RNA cleavage event in response to TFIIS with close to wild-type kinetics, but are unable to resume transcription past a block to elongation (12).

In yeast, the COOH-terminal two-thirds of TFIIS (residues 131–309) is sufficient for complete activity in vitro and in vivo (14). Structural analysis of this part of TFIIS shows that it comprises two stably folded domains, which are joined by a more mobile, yet essential linker region (15, 16). Our aim was

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*** The abbreviations used are: TFIIS, transcription elongation factor II; PCR, polymerase chain reaction; HMK, heart myosin kinase.
1 R. G. Weilbaecher and C. M. Kane, manuscript in preparation.
to determine how each of the domains of TFIIS contributes to function. To this end, we conducted structure-guided mutagenesis of the COOH-terminal two-thirds of yeast TFIIS to generate a comprehensive set of mutations and tested their effects in assays of transcription, RNA cleavage and RNA polymerase binding. These results reveal specific surfaces on the individual TFIIS domains involved in functional interactions and provide important details about the mechanisms of TFIIS-stimulated transcript elongation.

MATERIALS AND METHODS

Purification of Yeast RNA Polymerase II—RNA polymerase II was purified from baker’s yeast as described previously (17), except that 10 mM ZnCl2 was included in all buffers. The polymerase preparations were stored at -70 °C in 20 mM Tris-HCl, pH 7.9, 40 mM ammonium sulfate, 10% glycerol, 10 mM dithiothreitol, 1 mM EDTA, and 10 mM ZnCl2.

Purification of Yeast TFIIS—Yeast TFIIS was cloned and expressed as a fusion protein containing an amino-terminal hexahistidine tag in bacterial cells. All purification steps were performed at 4 °C. Cells containing TFIIS or derivatives were sonicated in Buffer A (20 mM Hepes pH 7.5, 10% glycerol, 10 mM ZnCl2, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine) containing 500 mM NaCl and 5 mM imidazole, clarified by centrifugation for 1 h at 100,000 × g at 4 °C and bound to His Bind resin (Novagen, WI). The column was washed with five column volumes of 60 mM imidazole in Buffer A and developed with a step elution of 500 mM imidazole in Buffer A. Mutants K196A, R198A, and R200A were purified using the protocol recommended by Novagen, with a 1 mM imidazole elution. The eluate from the His-Bind resin was dialyzed in Buffer A containing 50 mM NaCl and 10 mM dithiothreitol and stored at -70 °C. This expression and purification strategy resulted in a typical yield of 35 to 40 mg of TFIIS per liter of cell culture with an approximate purity of 85% TFIIS by mass (data not shown). Wild-type and mutant TFIIS proteins used in NMR experiments were purified as described previously (15).

Site-directed Mutagenesis of TFIIS—All TFIIS mutants except K196A, R198A, and R200A were constructed by PCR amplification of yeast TFIIS using oligonucleotides encoding the desired mutation. The three exceptions were generated using the method of Kunkel (18). For the PCR mutagenesis, two PCR products were produced, one containing a portion of the wild-type sequence and the other containing the remainder of the TFIIS sequence harboring mutations introduced with the mutagenic oligonucleotide. These PCR fragments were phosphorylated to introduce a 5′-phosphate, and then ligated using T4 ligase. The ligated products were used as the template in a second PCR reaction which produced the coding region for amino acid residues 131–309, containing the site-directed mutations, as well as NdeI and BamHI restriction sites at the 5′ and 3′ termini, respectively. The PCR products were digested with these restriction enzymes and ligated into pET 15bHMK, containing the heart myosin kinase consensus sequence. All plasmids were sequenced to verify the DNA sequence.

Binding Assay between Yeast TFIIS and RNA Polymerase II—The interaction between TFIIS and RNA polymerase II was investigated using a native gel mobility shift assay (10). TFIIS and derivatives were expressed as a fusion protein containing an hexameric histidine repeat followed by a thrombin site, the heart myosin kinase (HMK) consensus phosphorylation sequence, and the coding region of TFIIS. The fusion protein was constructed by inserting an oligonucleotide encoding the HMK consensus phosphorylation sequence (RRASVDF) into the NdeI site of the plasmid pET 15b, generating pET 15bHMK. The TFIIS coding region was inserted between the NdeI and BamHI sites (5′ and 3′, respectively) of pET 15bHMK and the resulting TFIIS fusion protein was expressed and purified as described above. The protein was phosphorylated at the HMK consensus site using HMK (Sigma) and [γ-32P]ATP according to the manufacturer’s instructions. The labeled protein was dialyzed against Buffer A containing 10 mM dithiothreitol and 50 mM NaCl to remove the free radiolabel. The 32P-labeled TFIIS and unphosphorylated TFIIS were equally active in assays of read-through and transcript cleavage. The TFIIS fusions were radiolabeled to an average specific activity of 4.5 x 106 cpm/pmol. The TFIIS molecules for the binding assay contained 20 mM Hepes pH 7.5, 10% glycerol, 10 mM ZnSO4, and 10 mM dithiothreitol. The binary TFIIS-RNA polymerase II complex was resolved from free yeast TFIIS by electrophoresis for 2 to 3 h at 100 volts at 4 °C on a 5% polyacrylamide (30:0.8 acrylamide: bisacrylamide) gel containing 50 mM Tris, 50 mM borate, 10 μM ZnSO4, and 1% glycerol at pH 8.3. The amount of bound 32P-HMK-TFIIS was quantified by PhosphorImaging and the binding constants estimated by half-maximal binding at equilibrium.

Transcript Elongation Assay: Read-through and Nucleolytic Cleavage by Arrested Ternary Complexes—The ability of TFIIS to stimulate arrested ternary complexes to transcribe through intrinsic arrest sites was analyzed by an in vitro transcript elongation assay. These procedures were carried out as described previously (12, 19). Briefly, transcript by purified RNA polymerase II was initiated from a 3′-deoxy-cytidine-tailed template containing a human histone H3.5 gene fragment, which contains well characterized blocks to elongation. The RNA transcript was labeled at the 5′ proximal end by incubating in the presence of [α-32P]CTP (3000 Ci/mmol, NEN Life Science Products Inc.), 0.8 mM GTP, ATP, and UTP for 75 s, followed by transcription to arrest sites in the presence of 0.1 mM unlabeled CTP for 75 s. Ternary complexes stalled at these sites were treated in two different ways. For the read-through assay, yeast TFIIS was added and transcription was allowed to proceed for the designated intervals. The resulting transcripts were collected by ethanol precipitation and resolved by electrophoresis on a 6% polyacrylamide gel (19:1 acrylamide: bisacrylamide) containing 8.3 M urea, 50 mM Tris, pH 8.3, 50 mM borate, and 0.1 mM EDTA. The transcripts were quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

For the TFIIS stimulated cleavage of the nascent RNA transcript, the stalled ternary complexes were purified from unincorporated nucleotides by two sequential Bio-Gel 30 spin columns (Bio-Rad, Mississauga, ON). TFIIS was then added to the purified complexes and incubation was continued at 30 °C. At designated time points, aliquots were removed and the transcripts resolved and quantified as described above.

NMR Analysis of TFIIS Mutants—15N-HSQC NMR spectra for each of the site-directed mutants was collected and analyzed as described previously (16).

RESULTS

A Role for the Region Linking the Three-helix Bundle and the Zinc Ribbon

The NMR analysis of TFIIS131–309 defined two ordered domains joined by an unstructured linker (16). Residues 131–240 comprise domain II, a three-helix bundle. Residues 240–260 correspond to the linker region. Residues 260–309 (domain III) comprise a zinc ribbon. It was known previously that the regions corresponding to the helix-bundle (domain II) and the zinc ribbon (domain III) are required for TFIIS transcription activity; however, the function of the linker region is unknown. To explore if the linker region is required for TFIIS activity, we tested if addition of a combination of purified domains II and III to a stalled transcription complex could reconstitute TFIIS activity. In assays of both transcript cleavage and transcription elongation, the purified domains were inactive, even when both were added in 500-fold molar excess over RNA polymerase II.

TFIIS131–240 Is the RNA Polymerase II-binding Domain

Our first aim was to determine which region of TFIIS mediated the interaction with RNA polymerase II. Both Horikoshi et al. (20) and Agarwal and colleagues (21) used glycerol gradient sedimentation to measure the interaction of radiolabeled TFIIS with RNA polymerase II. However, the same assay in two different labs pointed to two different regions of TFIIS. The conflicting results likely arose because of the non-quantitative nature of this assay. For example, in one of the glycerol gradient analyses that was reported (20), only a small percentage (less than 0.1%) of the input TFIIS bound to the polymerase and less than 1% of the polymerase molecules interacted with TFIIS. This analysis calls into question whether the published glycerol gradient analysis measured a specific, physiologically important interaction. Therefore additional studies were un-

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2 D. E. Awrey, unpublished data.
dertaken to identify the region of TFIIS that interacts with polymerase.

To identify the polymerase-binding domain on TFIIS, we developed an assay to measure the physiologically relevant interaction of TFIIS with RNA polymerase II. We used a gel mobility shift assay which exploited the great difference in mass and electrophoretic mobility between TFIIS and RNA polymerase (10). In this assay, TFIIS is radiolabeled through an amino-terminal kinase tag, which is a consensus phosphorylation site for heart myosin kinase. Next, the interaction of the two proteins is assayed by measuring the amount of TFIIS that co-migrates with the position of a TFIIS-RNA polymerase II complex following native gel electrophoresis.

The parameters of the assay were characterized using TFIIS131–309, which is completely active for transcription. TFIIS131–309 is positively charged; under the gel conditions, the unbound protein migrated out of the gel toward the cathode, and was not observable. Under the same conditions, RNA polymerase II migrated into the gel. When a constant amount of radiolabeled TFIIS was incubated with increasing amounts of RNA polymerase II, a radiolabeled species that migrated with the approximate position of RNA polymerase II was observed (Fig. 1). To confirm that this radiolabeled species was a complex of TFIIS and RNA polymerase II, we excised the corresponding region of the gel and analyzed its protein composition by denaturing gel electrophoresis and silver staining. All of the RNA polymerase II subunits and TFIIS co-migrated with the radiolabeled species, and neither the polymerase subunits nor TFIIS were found in any other part of the gel (data not shown). The measured binding reflects a specific, physiologically relevant interaction for the following reasons. First, the binding is saturable and stoichiometric (Fig. 1), and the interaction is both reversible and salt sensitive (data not shown). Second, the interaction was specific for yeast RNA polymerase II as opposed to the bacterial enzyme (data not shown). Third, with this assay, TFIIS did not interact with a point mutant RNA polymerase II, a mutant that is suppressed by overproduction of TFIIS in vivo (10).

Under the conditions of the gel mobility shift assay, TFIIS131–309 bound RNA polymerase II with an affinity of 58 nM. We tested the series of TFIIS deletion mutants for RNA polymerase binding. Neither domain I (residues 1–131) nor the zinc ribbon (residues 260–309) showed any binding to RNA polymerase in the gel shift assay, which is able to detect interactions with dissociation constants as weak as 1.5 μM (data not shown). Domain II alone (residues 131–240), however, interacted with a similar affinity (80 nM) as did TFIIS131–309 (Fig. 1, panel B). Domain II is therefore necessary and sufficient for RNA polymerase binding.

We also wished to compare the affinity of TFIIS131–309 and full-length TFIIS for RNA polymerase II. However, we could not accurately measure the affinity of full-length TFIIS because its electrophoretic properties caused it to be retained in the wells of the native gel.

Site-directed Mutagenesis

To gain more insight into the functionally important regions in TFIIS, we performed site-directed mutagenesis of the linker region and domains II and III, guided by our knowledge of the three-dimensional structure (15, 16). Solvent-exposed charged and aromatic residues were changed to alanine, either in clusters or individually. We also constructed both an insertion and deletion mutant between residues 240 and 245. Each of the site-directed mutants was purified to apparent homogeneity and tested in three assays of TFIIS function; binding to RNA polymerase II, cleavage of the nascent transcript in the elongating complex of TFIIS and RNA polymerase II, a mutant that is suppressed by overproduction of TFIIS in vivo (10).

FIG. 1. Binding assay between TFIIS deletion mutants and RNA polymerase II. A, autoradiogram of a gel mobility shift assay between radiolabeled TFIIS131–309 and RNA polymerase II. The binary complex of TFIIS131–309 and RNA polymerase II is identified to the left; uncomplexed TFIIS131–309 migrates toward the cathode, out of the gel wells. Lanes 1–10 contain 0, 0.08, 0.15, 0.31, 0.46, 0.69, 1.15, 1.54, 2.31, and 4.62 pmol of RNA polymerase II, respectively. All lanes contain 1 pmol of [32P-HMK]-TFIIS131–309. B, autoradiogram of a gel mobility shift assay between radiolabeled TFIIS131–240 and RNA polymerase II. The binary complex of TFIIS131–240 and RNA polymerase II is identified to the left; uncomplexed TFIIS131–240 migrates toward the anode, into the gel, and appears as a higher mobility diffuse band. Lanes 1–10 contain 0, 0.08, 0.15, 0.31, 0.46, 0.69, 1.15, 1.54, 2.31, and 4.62 pmol of RNA polymerase II, respectively. All lanes contain 1 pmol of [32P-HMK]-TFIIS131–240. C, binding assays were quantified by PhosphorImager and plotted graphically. Filled circles represent TFIIS131–309 and filled squares represent TFIIS131–240. Error bars represent the standard deviation of three binding experiments for TFIIS131–309 and four binding experiments for TFIIS131–240. D, autoradiogram of a gel mobility shift assay between radiolabeled R198A mutant of TFIIS131–309 and RNA polymerase II.
TABLE I

| Mutant         | Read-through | Cleavage | Binding $K_d$ |
|----------------|--------------|----------|--------------|
| 131–309        | 3            | 3        | ND           |
| 131–240        | 0            | 0        | 180          |
| 131–263        | 0            | 0        | 180          |
| 222–309        | 0            | 0        | ND           |
| 264–309        | 0            | 0        | >1500        |
| K154A/D158A    | 2            | 2        | 196          |
| K176A          | 2            | 2        | 34.4         |
| N187A/C188A/D189A | 2       | 2        | 56           |
| N191A/E192A    | 3            | 2        | 184          |
| K196A          | 2            | 2        | >1500        |
| R198A          | 2            | 2        | >1500        |
| R200A          | 2            | 1        | 1289         |
| R205A          | 2            | 2        | 1289         |
| N210A/N211A/D213A | 3     | 2        | 163          |
| N220A/D222A    | 3            | 2        | 97           |
| D232A/K234A    | 3            | 3        | 47           |
| D255A          | 3            | 3        | ND           |
| K242A/Q243A    | 2            | 2        | 142          |
| Insertion 240  | 0            | 0        | 80           |
| (LKQKI)        |              |          |              |
| Deletion 240–245| 0           | 0        | 83           |
| N252A/N255A/Q257A | 1     | 1        | 19.4         |
| T286A/D267A    | 2            | 2        | 16.3         |
| F269A          | 1            | 2        | ND           |
| K273A/K275A    | 2            | 2        | 76.4         |
| T286A/S288A    | 2            | 2        | 2.5          |
| R287Q          | 1            | 1        | 174.7        |
| F291N          | 0            | 0        | 79.8         |
| E291H          | 0            | 0        | 50.7         |
| R287Q/E291N    | 0            | 1        | 292          |
| R287Q/E291H    | 0            | 0        | 103          |
| R287Q/E291L    | 0            | 0        | 166          |
| F296A          | 1            | 2        | ND           |
| R305A          | 2            | 2        | ND           |
| K307A          | 0            | 2        | ND           |
| F308A          | 2            | 2        | ND           |

$^a$ ND, not determined.
$^b$ Read-through and cleavage were determined for the 1–309 mutant and binding for 131–309 mutant.

gation complex, and stimulating transcription through the histone H3.3 Tia intrinsic arrest site (read-through) (19). The activity in transcription read-through was standardized by the rate of disappearance of elongation complexes stalled at the T1a site upon incubation with a 5:1 molar ratio of TFIIS:RNA polymerase II (19). The activity in transcript cleavage was assessed by the rate of appearance of cleavage products, using a 5:1 molar ratio of TFIIS:RNA polymerase II, in the absence of nucleotides and under standard reactions conditions. The binding to RNA polymerase II was quantified using the native gel mobility shift assay. The list of mutations that were generated, and their activities, is shown in Table I.

**Structural Characterization of Site-directed Mutants**

Our alanine scan mutagenesis strategy, using the structure to specifically target surface residues, was not expected to cause any dramatic structural changes in TFIIS. However, we observed that a number of site-directed mutants behaved anomalously in our biochemical assays. To explore the possibility that these mutant proteins were misfolded, we evaluated the structural integrity of each of the suspect mutants by comparing the 15N-HSQC NMR spectra from each of the mutants with that of the wild-type protein. In this experiment, the pattern of peaks in the H praise the protein conformation. In this way, we were able to confirm that several mutants, D150A, K215A/K217A, Y281V/Y282, Q283A/Q285A, and Trp260 showed global structural defects (16). The lack of structural integrity compelled us to exclude all misfolded mutants from the analysis.

**TFIIS Residues Important for Mediating the Interaction with RNA Polymerase II**

Each of the properly folded site-directed mutants was tested for binding to RNA polymerase II. Four of the mutants, K196A, R198A, R200A, and K209A exhibited at least a 10-fold decrease in affinity for RNA polymerase II (Table I). These four critical residues localize to the outer face of the third helix in the three-helix bundle in domain II and the loop immediately COOH-terminal to this helix. To test if these residues define a charged surface of TFIIS, we examined the surface potential of the solvent accessible surface of domain II using the program, GRASP (22). This analysis revealed that the mutated residues define a basic patch on the surface of TFIIS (Fig. 2).

**Binding to RNA Polymerase II Is Required for Transcription Elongation Activity**

To test if binding of TFIIS to RNA polymerase II was required for transcription activity, each of the K196A, R198A, R200A, and K209A mutants was tested for the ability to promote transcript cleavage and to stimulate transcription through the human histone 3.3 Tia arrest site. The activity in transcription read-through was standardized by the rate of disappearance of elongation complexes stalled at the T1a site upon incubation with a 5:1 molar ratio of TFIIS:RNA polymerase II. Under these particular solution conditions, between 50 and 70% of the transcribing polymerases arrest at the T1a site and, in the course of a 30-min incubation with TFIIS, 70% of the arrested complexes are stimulated to read-through the T1a site (Fig. 3, panel A). TFIIS mutants were judged to be deficient in read-through if they effected a decrease in the percent of arrested complexes that could be stimulated to transcribe through the T1a pause site. The relative levels of activity between wild-type and mutant proteins were quantified by assessing the amount of TFIIS mutant needed to restore wild-type activity.

Transcript cleavage activity was standardized by the rate of appearance of cleavage products, using a 5:1 molar ratio of TFIIS:RNA polymerase II, in the absence of nucleotides and under standard reactions conditions. Specifically, there is a characteristic pattern of cleavage of the nascent transcript by arrested complexes at the T1a site. The first cleavage product, which is generated within minutes of incubation with TFIIS, is referred to as C1. The second cleavage product, C2, which is shorter, appears after a 5-min lag and is probably derived from the C1 product. Upon longer incubations with TFIIS, a range of much shorter transcripts are generated (Fig. 4). To compare the mutant and wild-type TFIIS molecules, we monitored the rate of appearance of the C1, C2, and smaller transcripts. TFIIS mutants were judged to be deficient in activity if either the rate or pattern of transcript cleavage was altered.

Transcript cleavage and read-through assays were performed for all of the TFIIS mutants defective in RNA polymerase II binding; the assays for a representative mutant (R198A) are shown in Figs. 3 and 4. Each of the mutants was unable to stimulate transcription read-through or cleavage when added in a 5-fold molar excess over the polymerase. To ensure that the defect in these mutants was due to an effect on polymerase binding and not to a defect in the stimulation of cleavage, the amount of TFIIS was increased to a 100-fold molar excess over RNA polymerase II. At these concentrations of TFIIS, which were predicted to saturate the binding to RNA polymerase II,
the binding mutants stimulated transcription to wild-type levels. Thus, these mutants are specifically deficient in binding to polymerase and probably have no defect in stimulating transcript cleavage. These results also suggest that binding to RNA polymerase II is the only transcriptional role for the three-helix bundle, and that the regions of TFIIS that stimulate cleavage and read-through are localized to the linker region and the zinc ribbon domain.

**The Linker Region**

Our mutagenesis of the linker region focused on the region that appears to have multiple conformations (residues 238–247) and the region that is more flexible (residues 250–263)(16). We also constructed both an insertion and deletion mutant between residues 240 and 245.

**Residues 238–247**—Residues 238–247 have NMR amide resonances too broad to observe experimentally (16). This property is consistent with these residues existing in dynamic equilibrium between two or more conformational states. To test the importance of this region for transcription, two of its charged residues, Lys242 and Gln243, were mutated to alanine and the resulting mutant protein (K242A/Q243A) assayed for function. The K242A/Q243A mutant bound RNA polymerase II with near wild-type affinity. However, both transcript cleavage and read-through activities were reduced 10- or 20-fold (Fig. 5).

To confirm the importance of this part of the linker region in the transcript cleavage and read-through, we generated a deletion mutant lacking residues 241–245 and a 5-residue insertion that duplicated the 240–245 region. Both mutants bound RNA polymerase II with normal affinity but had no read-through or cleavage activity (data not shown).

**Residues 250–264**—Residues 250–264 have narrow line widths and are more mobile compared with the rest of TFIIS (131–309) (16). We constructed a triple mutant of the charged residues within this region by mutating Asn252, Asn 255, and Gln257 to alanine. The resulting mutant bound RNA polymerase II with normal affinity (Table I). However, this mutant was dramatically impaired for both read-through and cleavage activities; activity could be observed only at 100 times higher concentration of TFIIS than was required to stimulate the wild-type protein (Fig. 6). Since we could not detect an effect of these mutations on the structure of domain II or the zinc ribbon, we conclude that this part of the linker region must exert its effect in the context of the TFIIS:RNA polymerase II elongation complex.

**Analysis of the Zinc Ribbon**

**The Aromatic Residues**—The zinc ribbon in yeast TFIIS contains six aromatic residues. We chose to mutate these residues for two reasons. First, unlike many other proteins, in which the aromatic residues are largely buried in the hydrophobic interior of the protein, in the TFIIS zinc ribbon, some aromatic
residues are exposed to solvent (16, 23). As such, these residues could potentially play a role in protein-protein interactions. Second, the zinc ribbon of TFIIS is suggested to bind nucleic acid, and in many single-stranded nucleic acid-binding proteins, solvent-exposed aromatic residues participate in base stacking interactions (24, 25).

Three mutants, Phe269, Phe296, and Phe308, remained folded after mutation to alanine, and each had decreased transcription activity. The F308A mutation was the least affected; wild-type read-through and cleavage activities were reduced 2–10-fold compared with the wild-type TFIIS. The F269A and F296A mutations had greater effects on TFIIS activity. Transcript cleavage activity was reduced 2–10-fold, and read-through activity 20–100-fold. The results for the F296A mutant are shown in Fig. 7.

The Charged Residues—The loop that connects the first and second strands of the β-sheet is disordered and contains several charged residues. The sequence, QTRSADEP, is conserved from yeast to man. Agarwal and colleagues (26) first demonstrated the requirement for the DE dipeptide in human TFIIS. All mutations in either amino acid completely destroyed TFIIS activity. The requirement for these residues for human TFIIS is illustrated in Fig. 7.

![Fig. 4. Analysis of TFIIS mutant R198A in transcript cleavage.](image)

![Fig. 5. Analysis of TFIIS mutant K242A/Q243A in transcript elongation assays.](image)
activity was also observed by Cipres-Palacin and Kane (27).

To confirm that the DE dipeptide was important in the context of the yeast TFIIS, Glu291 was mutated to either Asn or His. As expected, all of the mutants were completely inactive for cleavage and read-through (data not shown), although each bound RNA polymerase II with wild-type affinity. Agarwal and colleagues (26) also showed that mutations of other amino acids in this loop inactivated TFIIS. To explore the roles of the rest of the loop in yeast TFIIS, two other mutations were analyzed, R287Q and T286A/S288A. The T286A/S288A mutant showed only mildly reduced activity although it bound RNA polymerase to a slightly better than wild-type. Mutation of this residue in the human TFIIS also decreased TFIIS activity (26).

Several double mutations of Arg287 and Glu291 were constructed. Although every mutation of Glu291 was inactive, the
and the loop immediately COOH-terminal to this helix (residues 205–215).

Our results provide a plausible molecular mechanism for the phenotype of mutations of human TFIIS that were previously reported by Ciprés-Palacín and Kane (13, 27), who used alanine scanning mutagenesis to evaluate the importance of charged residues in human domain II (27). Our data suggest that some of the mutations that were studied were probably misfolded and another was likely deficient in binding to RNA polymerase.

In their study, 11 mutations were made in the domain II region. Three clusters of mutations (clusters 1, 7, and 7b) dramatically decreased or eliminated transcription activity. Mutations in either cluster 1 (a quadruple mutant, R141A/K143A/R145A/E146A) or cluster 7b (a triple mutant, R190A/R192A/R194A) abolished both read-through and transcript cleavage activities. Mutation of cluster 7 (containing mutations K186A/K188A) decreased transcript cleavage activity two-fold, but abolished read-through activity.

The amino acid residues altered in cluster 1 correspond to the region of yeast TFIIS containing the site-directed mutant D150A. In the three-dimensional structure of domain II, the Asp150 side chain interacts with the His145 side chain and probably stabilizes the packing of the NH2-terminal β-strand (131–143) to the first α-helix of the three-helix bundle. The HSQC spectra of the D150A mutant is considerably different from that of the wild-type protein, suggesting that the D150A mutant is misfolded. We suggest that the cluster 1 mutations in human TFIIS disrupted its structure and hence generated an inactive protein. Consistent with this hypothesis was the observation that this human mutant “was recovered as a smaller fraction of total protein” following overexpression compared with the other human mutants (27). This retrospective re-interpretation of the alanine scanning mutagenesis data underscores the importance of analyzing any mutant within a structural context.

The human cluster 7b mutations correspond to the yeast mutants R198A and R200A, which display a reduced affinity for RNA polymerase II. These yeast site-directed mutants display small structural alterations, as judged by NMR analysis, indicating the introduction of these mutations does not affect the protein structure. Thus, it is likely that the inactivity of the human cluster 7b mutant is caused by a reduced affinity of this mutant for RNA polymerase II.

Mutations in a third cluster within domain II, cluster 7, retained some cleavage activity but lost read-through activity (13). Specifically, the cluster 7 mutant stimulated the appearance of 50% of the amount of the TII transcripts as did the wild-type protein. This human TFIIS mutant containing the cluster 7 alterations corresponds to the yeast site-directed mutant K196A, which displayed a reduced affinity for RNA polymerase II. Based on the sequence homology between yeast and human TFIIS, the human cluster 7 mutant probably has a reduced affinity for RNA polymerase II. It is not obvious why the human cluster 7 mutant retained some activity while the clusters 1 and 7b mutants did not. Perhaps this mutant retains enough binding activity to facilitate the initial cleavage event, but the interaction was too weak to efficiently allow the subsequent TFIIS-dependent process that is required for read-through.

The interaction of TFIIS with RNA polymerase II is known to be sensitive to high ionic strength (28), suggesting that the interaction is ionic in character. Our molecular characterization of the interaction interface is in keeping with these observations. We showed here that a basic patch of TFIIS mediates the interaction with RNA polymerase II. Our previous analysis of RNA polymerase II mutants suggested that the domain that

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**Fig. 8. Analysis of TFIIS mutant R287Q/E291N in transcript elongation assays.** A, graphical representation of PhosphorImager quantified TII and run-off transcript products prepared and resolved as described under “Materials and Methods.” % at TII is as described in the text (TII/(TII+RO) × 100%). Diamonds represent products of incubation with 5:1 molar ratio; and triangles represent the products of 100:1 molar ratio of TFIIS131–309 (R287Q/E291N):RNA polymerase II. B, transcript cleavage products were obtained and resolved as described under “Materials and Methods” and visualized by autoradiography. Arrosted ternary complexes were stimulated with the indicated amounts of TFIIS131–309 and R287Q/E291N for the designated times. Nucleotides were added in the indicated lanes and incubation allowed to proceed for an additional 10 min. The positions of the TII, TII, C1, and C2 cleavage sites, and the run-off transcript (RO) are indicated.

R287Q/E291N double mutant displayed some cleavage activity, when added in 500-fold molar excess over RNA polymerase (Fig. 8).

**DISCUSSION**

**RNA Polymerase II Binding**—The three-helix bundle of yeast TFIIS is necessary and sufficient for the interaction of TFIIS with RNA polymerase II. Each of the following mutations, K196A, R198A, R200A, and K209A, reduced the affinity of the interaction between TFIIS131–309 and RNA polymerase II at least 10-fold without affecting the global structure of TFIIS. The solution structure of both TFIIS131–240 and TFIIS131–309 revealed that the residues implicated in binding to RNA polymerase are localized to a basic, solvent accessible surface of the third helix (amino acids 193–204) of the three-helix bundle and the loop immediately COOH-terminal to this helix (residues 205–215).

Our results provide a plausible molecular mechanism for the phenotype of mutations of human TFIIS that were previously reported by Ciprés-Palacín and Kane (13, 27), who used alanine scanning mutagenesis to evaluate the importance of charged residues in human domain II (27). Our data suggest that some of the mutations that were studied were probably misfolded and another was likely deficient in binding to RNA polymerase. In their study, 11 mutations were made in the domain II region. Three clusters of mutations (clusters 1, 7, and 7b) dramatically decreased or eliminated transcription activity. Mutations in either cluster 1 (a quadruple mutant, R141A/K143A/R145A/E146A) or cluster 7b (a triple mutant, R190A/R192A/R194A) abolished both read-through and transcript cleavage activities. Mutation of cluster 7 (containing mutations K186A/K188A) decreased transcript cleavage activity two-fold, but abolished read-through activity.

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interacts with TFIIS is acidic (10); one of the point mutants in RNA polymerase that fails to interact with TFIIS contains a change of glutamic acid to lysine. We suggest that this mutation defines an acidic patch on RNA polymerase that interacts with TFIIS.

The linker Region—Although the linker region appears largely unstructured, for two reasons we believe that it is not simply a flexible spacer between the two structured domains. First, the linker region has some stretches that adopt multiple conformations (16). Second, a few site-directed mutants in the linker region destroyed activity, even though these mutants did not affect the structures of either domain II or the zinc ribbon. If the role of the linker region was to simply tether domain II with the zinc ribbon, it is unlikely that mutating two amino acids would affect activity. We consider it more likely that the linker region adopts a crucial structure in the ternary elongation complex. Our mutagenesis experiments suggest that the regions spanning residues 240–245 and 250–263 are critical.

The Zinc Ribbon—Domain III, which is composed of residues 260–309, contains the zinc ribbon structural motif that is required for TFIIS-mediated transcript cleavage. Domain III is apparently not required for the interaction with RNA polymerase II; no mutants in the zinc ribbon decreased RNA polymerase II binding and no interaction between the purified zinc ribbon and RNA polymerase was detected in native gel mobility shift assays, which have a sensitivity of 1.5 μM.

Many mutations in domain III affected transcript cleavage and read-through activities without affecting the overall structure of TFIIS. Site-directed mutants F269A, T286A/S288A, R287Q/E291N, F296A, K307A, and F308A had reduced transcript cleavage and read-through activities. Site-directed mutants E291N, E291H, and R287Q/E291H were completely defective in stimulating transcription through the T1a arrest site. These two groups of mutations defined two parts of the zinc ribbon required for activity; a face of the β-sheet and the acidic linker that joins the first and second β-strands, respectively.

The importance of the acidic residues within the zinc ribbon of yeast TFIIS for the stimulation of transcript cleavage is supported by previous studies on a human TFIIS homologue. Site-directed mutations targeting conserved residues, from 256 to 264, contained within the zinc ribbon of human TFIIS (which correspond to residues 285 to 293 of the yeast homologue) were demonstrated to abrogate transcript cleavage activity (26, 27). The mutations introduced into the yeast TFIIS homologue at these same conserved residues has the same effect.

The region of human TFIIS homologous to domain III and the linker region has been shown to interact with single-stranded and duplex nucleic acids (26). This interaction is observed only in the absence of domains I and II, and the affinity between domain III and nucleic acid is low with a Kd near 10^{-8} M. The interaction of TFIIS with nucleic acid is also supported by the observation that TFIIS can be cross-linked to the nascent RNA within a stalled elongation complex (29).

We have not detected a reproducible interaction of domain III with nucleic acid using gel mobility shift studies. Although we have tested each of our mutations in domain III (in the context of purified domain III) for nucleic acid binding using either electrophoretic mobility shift assays or fluorescence anisotropy, the results from each technique were too equivocal to interpret. Nevertheless, the putative nucleic acid interaction, although virtually undetectable in vitro, may be important in the context of the ternary complex, in which the local concentration of nucleic acid is high. Clearly, resolving the nucleic acid binding issue is of paramount importance.

Structural Similarity of the Zinc Ribbon to an RNA Binding Motif Supports Putative Nucleic Acid Binding Activity—RNA-binding motifs, both oligosaccharide and oligonucleotide-binding fold and RNA-binding domains, are composed of a β-sheet region upon which the RNA bases are splayed (24, 25, 30). A conserved feature among RNA-binding domains is a set of aromatic residues that protrude from the β-sheet and are solvent accessible. The exposed aromatic residues are important for interacting with the RNA strand by stacking with the nucleotide bases (24, 25). Site-directed mutagenesis of solvent-exposed aromatic residues in TFIIS (for example, F296A) caused a reduction in TFIIS activity without destabilizing the structure of the zinc ribbon. Several aromatic residues lie on the surface of the β-sheet and are conserved in TFIIS homo-

**FIG. 9.** Model for zinc ribbon nucleic acid binding activity. Ribbon diagrams showing the structures of a single-stranded DNA-binding domain of human replication protein A (25) bound to a dC trinucleotide (left panel) and the zinc ribbon from yeast TFIIS (right panel). β-Strands are shown as green arrows. The zinc atom in TFIIS is shown in red. The DNA in the left panel is shown in pink. Aromatic side chains that form stacking interactions in replication protein A and the putative corresponding residues in TFIIS are shown in blue.
logues (Fig. 9). Based on this analogy, TFIIS may bind to the nascent RNA transcript or the single-stranded DNA in the transcription bubble using stacking interactions.

**Transcript Cleavage at the Tta Site: Two Mechanistically Different Events**—Reines and colleagues (31, 32) demonstrated that two major cleavage products are generated by mammalian transcription complexes stalled at the Tta site. In the yeast system, we observe the same cleavage pattern and refer to these sites as C1 and C2. Reines and colleagues (32) showed quite conclusively that only one cleavage event, at the C1 site, was necessary for progression through the Tta arrest site. However, in our analysis of several RNA polymerase II and TFIIS mutants, the appearance of the C1 transcript did not always correlate with reactivation of the stalled complex to read through the arrest site. In fact, the ability to read-through was more highly correlated with the ability to generate the C2 cleavage product.

Our model to explain this apparent paradox emerges from current ideas about the structure of an arrested elongation complex. Transcription arrest is thought to involve at least two steps. In the first step, the elongating RNA polymerase encounters some form of block that momentarily pauses its forward movement. In the case of the Tta site, pausing is initiated by interactions between RNA polymerase II and a specific DNA sequence or structure. In the second step, which transforms the paused complex to an arrested complex, the polymerase active site loses register with the paused complex to an arrested complex, the polymerase active sequence or structure. In the second step, which transforms the paused complex to an arrested complex, the polymerase active site loses register with the 3′ end of the transcript and probably slips back a few nucleotides (2). The final arrested complex is therefore maintained in two ways. First, the 3′-end of the RNA, which is now dissociated from the active site, inhibits nucleotide incorporation. Second, specific RNA polymerase-nucleic acid interactions inhibit translocation of the enzyme. Thus, we suggest that, just as the process of arrest involves two steps, TFIIS-mediated reactivation of arrested complexes involves two steps: transcript cleavage and conversion of the translocation-incompetent complex to a translocation-competent complex.

We suggest that cleavage to the C1 site is the first of two TFIIS dependent processes that are necessary for the reactivation of stalled complexes. The second step involves the conversion of the translocation-incompetent complex to one that is translocation competent. This conversion allows the ternary complex to translocate forward past the arrest site. There is no requirement for the C2 cleavage event in this model. We suggest that the C2 product appears only as a consequence of incubating translocation-competent complexes in the absence of nucleotides.

If our model is correct, the C2 transcript must be derived from the C1 transcript. Although we have not conclusively demonstrated this, for three reasons, it is likely to be true. First, in TFIIS mutants displaying a reduced rate of transcript cleavage, near quantitative transcript cleavage occurs at the first cleavage site prior to the second cleavage event. Second, the formation of the C2 transcript causes a concomitant decrease in the amount of C1, without affecting the residual amount of arrested complex at Tta. Finally, C2 never appears in the absence of C1.

**Model of TFIIS Action**—The initial step in the activation of arrested complexes is most likely the binding of TFIIS to RNA polymerase II. From our studies on the TFIIS-RNA polymerase II interaction, we have been able to localize the interacting regions to the third helix of TFIIS domain II and the spacer between conserved regions G and H of the largest subunit of RNA polymerase II (10). The minimal amount of TFIIS required to maximally activate transcription is the same amount required to saturate binding to RNA polymerase II. We infer from this that TFIIS interacts with the free RNA polymerase and the ternary complex with equal affinity. It is therefore unlikely that any other part of TFIIS contributes greatly to the interaction. In the arrested complex, there is likely a structural alteration of the RNA-DNA interaction that leads to the release of the 3′-end of the nascent RNA transcript (2, 3). We propose that the second event in the reactivation process is the recognition, or binding, of this free 3′-end of the nascent RNA by the TFIIS zinc ribbon domain, which has the structural hallmarks of a single-stranded nucleic acid-binding domain. In keeping with the structural similarity with other nucleic acid-binding proteins (24, 25, 30), we speculate that the free bases of the 3′-end of nascent RNA are splayed across the β-sheet of the zinc ribbon, stacking with the solvent-exposed Phe296 residue. The interaction of the RNA chain with the zinc ribbon distorts the C1 cleavage site near the polymerase active site. Domain III has an additional role in transcript cleavage. We speculate that the acidic residues within the disordered loop of the zinc ribbon play a base catalysis role, deprotonating the nucleophilic residue(s) in RNA polymerase that will cleave the RNA chain. The ability of the ternary complex to cleave the nascent transcript under alkaline conditions indicates that the deprotonation event may occur at a residue located near the catalytic site of the polymerase.

Our data suggest that at this stage of the activation cycle, a second TFIIS stimulatory event is required. We have isolated mutants of both RNA polymerase and TFIIS that can perform the first cleavage step but are unable to continue transcript elongation (12, 13). We propose that after transcript cleavage, a conformational change in the ternary complex is required prior to the resumption of transcript elongation, and that this conformational change converts the arrested complex to one that is translocation competent. We are unable to offer a molecular explanation for the conformational change, but can say that the change is influenced by TFIIS and at least one other peptide, one of the smaller RNA polymerase II subunits, RPB9 (12). Our current model is that the arrested complex is stabilized by a specific interaction of the polymerase with the DNA, and that TFIIS and possibly other proteins play a role in facilitating the dissociation of the polymerase from binding the nucleic acid in the arrest mode, allowing the polymerase to continue elongation.

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