Yeast Transcription Elongation Factor (TFIIS), Structure and Function

I. NMR STRUCTURAL ANALYSIS OF THE MINIMAL TRANSCRIPTIONALLY ACTIVE REGION*

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TFIIS is a general transcription elongation factor that helps arrested RNA polymerase II elongation complexes resume transcription. We have previously shown that yeast TFIIS (yTFIIS) comprises three structural domains (I-III). The three-dimensional structures of domain II and part of domain III have been previously reported, but neither domain can autonomously stimulate transcription elongation. Here we report the NMR structural analysis of residues 131–309 of yTFIIS which retains full activity and contains all of domains II and III. We confirm that the structure of domain II in the context of fully active yTFIIS is the same as that determined previously for a shorter construct. We have determined the structure of the C-terminal zinc ribbon domain of active yTFIIS and shown that it is similar to that reported for a shorter construct of human TFIIS. The region linking domain II with the zinc ribbon of domain III appears to be conformationally flexible and does not adopt a single defined tertiary structure. NMR analysis of inactive mutants of yTFIIS support a role for the linker region in interactions with the transcription elongation complex.

The rate of transcription elongation by RNA polymerase II is regulated by elongation factors, of which there are two classes with different mechanisms of action. One class, which includes elongin, ELL, pTEFb, and RAP74 stimulates the rate of nucleotide incorporation. The other class, includes TFIIS, its viral and archaeal sequence homologues, and the bacterial proteins, greA and greB. These proteins do not affect the rate of nucleotide incorporation, but rather stimulate an activity in RNA polymerase II that enables it to transcribe through DNA that contains single-stranded DNA (4, 9). The structures of yeast domain II and domain III of human TFIIS have been solved individually. However, these proteins are unknown, however, in TFIIS it has been suggested to be involved in binding to both single- and double-stranded DNA.

Although TFIIS1 and its bacterial homologues have very similar mechanisms of action, their primary sequences and three-dimensional structures are quite different. GreA and greB each comprise 160 amino acids that form two 80-residue structural domains. In greA, the N-terminal domain is composed of two extended, coiled antiparallel α-helices, and can be cross-linked to the nascent RNA when bound to RNA polymerase II in a stalled elongation complex (2). The C-terminal domain of greA is globular and is composed of a β-sheet that cradles an α-helix. GreB is a sequence homologue of greA and likely has an identical tertiary structure. yTFIIS is composed of three domains (domains I, II, and III) as defined by limited proteolysis and structural studies using nuclear magnetic resonance (NMR) spectroscopy (3) (see Fig. 1). Domains II and III, which together extend from residues 131–309 in yeast, are sufficient for transcription activity. Domain II (residues 131–240 in yeast) contains a three-helix bundle (3) and domain III is a zinc ribbon, which contains a three-stranded β-sheet, stabilized by a tetrad of cysteine residues that chelate a zinc ion (4). Domain II is conserved in TFIIS homologues and is postulated to interact with RNA polymerase II (4). Domain III is also conserved among TFIIS homologues, and possesses a structural homologue in TFIIB (5) and sequence homologues in RPB9 (the ninth largest subunit of RNA polymerase II) (6), TFIIE (7), DNA polymerase α (8), and T4 DNA primase (GenBank accession number 118732). The function of the zinc ribbon in each of these proteins is unknown, however, in TFIIS it has been suggested to be involved in binding to both single- and double-stranded nucleic acid (4, 9).

The structures of yeast domain II and domain III of human TFIIS have been solved individually. However, these proteins individually or in combination fail to stimulate transcription elongation. Furthermore, mutations at the junction between domain II and III in human TFIIS can abrogate elongation activity (10) suggesting an important role for the region linking the two domains. Our aim in this study was to determine the structure of a transcriptionally active yTFIIS fragment. We have mapped the minimal fragment of yTFIIS that retains its structural integrity and full activity to residues 131–309 which comprises all of domains II and III. Our analysis of yTFIIS131–309 had three objectives. First, we wished to confirm that the structures previously determined for the isolated domains accurately reflected the structure of the same region in the transcriptionally active protein. Second, we wished to determine the structure of the 60-amino acid region that links the three-helix bundle of domain II with the zinc ribbon of domain III and determine the relative orientation, if any, of these two domains.

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† The abbreviations used are: TFIIS, transcription elongation factor TFIIS; PCR, polymerase chain reaction; NOE, nuclear Overhauser effect.
Third, we wished to use structural information to design site-directed mutants of yTFIIS, which were destined for use in exploring the function of each of the yTFIIS domains.

**MATERIALS AND METHODS**

**Cloning and Site-directed Mutagenesis of yTFIIS**—Sequences coding for truncation mutants of yTFIIS were cloned into the T7 polymerase expression vector pET15b (Novagen), as described in Morin et al. (3) and expressed as C-terminal fusions to an N-terminal 6-histidine tag and a thrombin protease site. yTFIIS point mutants were constructed by PCR amplification of yeast TFIIS using oligonucleotides encoding the desired mutation. For each mutant constructed, two PCR products were produced, one containing a portion of the wild-type sequence and the other containing the remainder of the yTFIIS sequence harboring the mutations introduced with the mutagenic oligonucleotide. These PCR fragments were phosphorylated to introduce a 5'-phosphate, and subsequently 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 Ndel 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 derived plasmids were sequenced to verify the DNA sequence and ensure the introduction of mutations.

**Purification of Recombinant yTFIIS**—yTFIIS131–309, and its point and truncation mutants were purified as described by Morin et al. (3). Transcript read-through assays were performed as outlined previously (11). yTFIIS264–309 expressed at much lower levels and was prepared as described by Farrow et al. (30), at 25 °C. Two-dimensional spectra were acquired with relaxation delays of 10, 16, 24, 71, 75, 222 ms for T1, and 17, 33, 50, 66, 83, 99, 116, and 132 ms for T2. Peak intensities were quantified using the program PIPP (14) and relaxation constants were determined by fitting a single exponential to the peak intensities. Steady-state heteronuclear NOE values were obtained by taking the ratio of the peak intensities from spectra recorded with and without 1H saturation applied before the start of the experiment. These spectra were obtained using yTFIIS264–309 with natural abundance isotopic composition. A two-dimensional NOESS (26) was run at 25 °C with a mixing time of 120 ms. The sample was lyophilized and resuspended in 99.996% D2O. This sample was used to acquire a second NOESS spectrum (mixing time = 120 ms) and a two-dimensional TOCSY (27) with mixing time of 38 ms.

**Structure Determination of yTFIIS264–309**—NOE data from both the 131–309 construct and the 264–309 construct were used to derive hydrogen-hydrogen distance restraints. These were classified as strong, medium, or weak intensity NOEs corresponding to distance ranges of 1.7–2.8 Å, 1.8–3.5 Å, and 1.8–5.0 Å, respectively. Pseudo-atom corrections were made to distance upper bounds for protons in methyl, methylene, and aromatic ring groups (31, 32). Since the geometry of the zinc ion coordination in yeast TFIIS was expected to be the same as for human TFIIS, we used distance and angle restraints reported for the human structure (33) as follows. The S-Zn distances of the coordinating cysteines, Cys271, Cys274, Cys299, and Cys302 were set to 1.7–2.8 Å and the S-Zn-S bond angles were restrained to the corresponding angles reported for the human protein, but with upper and lower bounds of ±8°. Characteristic interstrand NOEs and chemical shift data indicated that residues 279–285, 293–299, and 304–309 were in β-strand conformations. Thus, for each of these residues the φ dihedral angle was restrained to −120 ± 30°, and distance restraints were used for the cross-strand hydrogen bonds of this β-sheet (O-H distance = 1.8–2.4 Å, O-N distance = 2.7–3.3 Å). Three-dimensional structures were calculated by using these restraints (summarized in Table I) and the dynamic simulated annealing protocol implemented within X-PLOVer.
The structure of yTFIIS$_{131-309}$ was analyzed using two- and three-dimensional triple-resonance and isotope-edited NMR techniques. Due to the tendency of this protein to unfold in solution, the low signal to noise and complete resonance assignments could not be made for all residues. Nevertheless, our NMR analysis confirmed that the 3-helix bundle and zinc ribbon structures are present in yTFIIS$_{131-309}$ and are essentially the same as those determined for the isolated domains. The residues linking the 3-helix bundle with the zinc ribbon do not have a single defined conformation and therefore, additional data such as NMR relaxation experiments and NMR analysis of point mutants were collected in order to better understand the structural and dynamic relationship between the 3-helix bundle, the zinc ribbon, and the residues linking them.

Table II

| Deletion mutant | Read through activity$^a$ |
|-----------------|--------------------------|
| 113–309         | + + +                    |
| 131–309         | + + +                    |
| 138–309         | + + +                    |
| 144–309         | + + +                    |
| 149–309         | –                        |
| 192–309         | –                        |
| 186–309         | –                        |
| 222–309         | –                        |
| 264–309         | –                        |
| 264–309         | –                        |
| 114–224         | –                        |
| 114–240         | –                        |
| 131–224         | –                        |
| 131–240         | –                        |
| 131–263         | –                        |
| 144–224         | –                        |
| 144–240         | –                        |
| 131–309 (ins. @ 240: LKQKI) | – |
| 113–309 (del. @ 240–245: LKQKI) | – |

$^a$ The read through activity of mutants is relative to wild type yTFIIS activity: –, inactive; +++, 60–100% activity.

Mapping of the Minimal Transcriptionally Active Region

Solution structures have previously been reported for the three-helix bundle of domain II of yeast TFIIS (3) and the zinc ribbon of domain III of human TFIIS (9, 33). Individually, these domains are inactive for stimulation of transcript elongation. Our goal was to analyze the structure-function relationship of a transcriptionally active yTFIIS fragment. To identify a suitable fragment for structural studies, we purified many different N- and C-terminal deletions of yTFIIS and assayed their activity in transcript elongation assays (Table II). Any deletion from the C terminus abrogated activity, but N-terminal truncations up to residue 144 remained fully active. The 143–309 fragment was analyzed by NMR, but proved unsuitable for structure determination because the spectra showed evidence of conformational heterogeneity. However, a fragment containing residues 131–309, yTFIIS$_{131-309}$, was fully active for transcription activity and had a good quality $^{15}$N-HSQC NMR spectrum (Fig. 2), indicating that it was appropriate for structural analysis in solution. The improved stability of this fragment likely results from the presence of the N-terminal extended strand, residues 137–142, which packs against the first helix of the three-helix bundle of domain II (3).

**RESULTS**

**Mapping of the Minimal Transcriptionally Active Region**

The structure of yTFIIS$_{131-309}$ was conserved in the yTFIIS$_{264-309}$ fragment and, if so, whether the three-helix bundle interacted with other parts of yTFIIS. To address these questions, the backbone and $C^\beta$ chemical shifts of the residues in the three-helix bundle were compared for the yTFIIS$_{131-240}$ and yTFIIS$_{131-309}$ constructs. As shown in Fig. 3A, the difference in backbone amide chemical shift values for the two constructs were negligible, as were values for side chain resonances (data not shown). Because the NMR chemical shift is sensitive to changes in protein conformation, this close correspondence indicates that the structure of the three-helix bundle is the same in both yTFIIS$_{131-240}$ and yTFIIS$_{131-309}$. Because chemical shift values also reflect the local chemical environment of each nucleus, the data also demonstrate that the three-helix bundle is not tightly associated with domain III of yTFIIS.

The C-terminal portion of domain II in yTFIIS$_{131-240}$ had two regions, from residues 215–220 and 227–234, that have backbone chemical shifts consistent with an $\alpha$-helical conformation and the typical sequential and medium-range NOEs that are the hallmarks of an $\alpha$-helix. In the context of yTFIIS$_{264-309}$, this region had similar helical propensity based on chemical shift values. However, in neither structure did we observe any long range NOEs that would associate these two helices with each other or the three-helix bundle or zinc ribbon.

**Structure of the Zinc Ribbon**—The C-terminal region of yTFIIS$_{131-309}$ adopts a zinc ribbon structure very similar to the zinc ribbons reported for human TFIIS (9, 33) and TFIIB from Pyrococcus furiosus (5). Comparison of the NMR chemical shifts of yTFIIS$_{264-309}$ with those of the same residues in yTFIIS$_{131-309}$ showed that this region retains the same structure in both protein constructs and is therefore not dependent on residues N-terminal to it in the solution (Fig. 3B). The three-dimensional structure determined from data gathered on both proteins consists of a three-stranded anti-parallel $\beta$-sheet composed of residues 279–285 ($\beta_1$), 293–299 ($\beta_2$), and 304–308.
The Region Linking Domain II and the Zinc Ribbon—Residues 238–247 span the proteolytically defined boundary between domains II and III (3). We were unable to assign the resonances of these residues in yTFIIS_{131–309} due to lack of observable correlations in \(^{15}\)N-edited experiments. We interpret the absence of observable amide resonances for these residues as being due to conformational exchange between two or more states resulting in peaks that are too broad to detect. This may explain the proteolytic susceptibility of this region (3). Residues 248–263 have strong correlations in \(^{15}\)N-edited and triple resonance experiments, but do not have regular secondary structure as judged by NOEs or CSI analysis. Thus, the chemical shift and NOE data suggest that the N-terminal bundle and zinc ribbon domains are linked by residues that do not have a single defined tertiary, and in some regions, secondary structure. However, lack of NOE distance restraints per se does not necessarily demonstrate motional flexibility.

Dynamic Properties of yTFIIS_{131–309}

In order to better understand the role of the region linking the 3-helix bundle with the zinc ribbon, as well as the motional and spatial relationship between these domains, we measured \(^{15}\)N NMR relaxation parameters for yTFIIS_{131–309}. The \(^{15}\)N-\(T_1\), \(T_2\), and \(^{1}H-{^{15}}N\) heteronuclear NOE values of backbone amide nitrogens are dependent on the motions of the protein backbone and therefore reflect the dynamic behavior of the molecule or its domains in solution and can reveal differences in segmental mobility (37, 38). Fig. 5 shows a profile of the relaxation parameters measured for each backbone residue. The uniform values for residues within domain II suggest that the 3-helix bundle, together with the C-terminal region of domain II tumble in solution as a single domain. Similarly the values for the zinc ribbon region are also fairly uniform, but on average, slightly lower for \(T_1\) and slightly higher for \(T_2\) than those of domain II. Residues 248–263, on the other hand, have \(T_2\) and heteronuclear NOE values significantly different from those of either domain II or the zinc ribbon. These values for the linker region are suggestive of increased flexibility, relative to the other two domains, however, the significant positive value of the heteronuclear NOE for the linker also indicates that it has restricted motion compared with a fully flexible linker such as that observed in calmodulin (39). A more detailed analysis of the dynamics will be presented elsewhere, but this preliminary analysis suggests that the linker region may act as a semi-rigid spacer connecting domain II with the zinc ribbon.

Structure-based Analysis of Point Mutants

In order to better understand the role of the linker region in yTFIIS function and to see whether it is structurally coupled to either domain II or the zinc ribbon, we acquired \(^{15}\)N-HSQC NMR spectra of a series of yTFIIS_{131–309} mutants that were defective for transcription elongation activity. Fig. 6 shows a summary of a qualitative comparison of the spectra of each mutant with that of wild-type yTFIIS_{131–309}. Mutants in the linker region such as K242A/Q243A, N252A/N255A/Q257A, T266A/D267A, and LKQKI have only minimal changes in chemical shift compared with wild-type yTFIIS and therefore have conformations equivalent to that of wild-type yTFIIS_{131–309}. We were particularly interested to see the effects of the LKQKI mutation in which a repeat of the sequence between residues 241 and 245 was inserted between residues 240 and 241. Since we were unable to assign the resonances of residues 240–248 in wild-type yTFIIS_{131–309} (presumably due to conformational heterogeneity) we expected that the insertion mutant would result in either shifted resonances or a new set of peaks that might help us identify the signals of the unassigned residues. However, as shown in Fig. 6 the HSQC spectrum of LKQKI was virtually identical to that of wild-type yTFIIS_{131–309}.
suggesting that the inserted residues also undergo some type of conformational averaging which obscures their NMR signals. The fact that mutations in the linker region only affect the chemical shifts of residues immediately adjacent to the mutation in the primary sequence is consistent with a linker that lacks tertiary structure and is not associated with either the 3-helix bundle or the zinc ribbon.

The remaining mutants, D150A, K215A/K217A, Y281A/Y282A, Q283/Q285, and W306A have more extensive effects on the chemical shifts. For example, D150A and W306A have significant changes in chemical shifts for residues throughout the 3-helix bundle and zinc ribbon domains, respectively. Asp150 interacts with His145 in the turn between the N-terminal extended strand (Asp137-Ala142) and the first helix of the 3-helix bundle. This interaction probably stabilizes the packing of the strand against the helix and possibly stabilizes the helix itself. Truncation mutants that retain residue 145 are fully active, but truncations beyond residue 145 are inactive (Table II). Similarly mutation of Trp306 to alanine removes important hydrophobic interactions between β-strands, and from Trp306 to the N terminus of yTFIIS (35). Importantly, mutations that disrupt the 3-helix bundle do not disturb residues in the remainder of the protein and mutations that disrupt the zinc ribbon are for the most part limited to the zinc ribbon region. This result is further evidence that the 3-helix bundle and the zinc ribbon are structurally independent domains in the context of transcriptionally active yTFIIS. Comparison of the profiles for D150A and K215A/K217A in Fig. 6 indicates that the C-terminal region of domain II is not structurally coupled to the 3-helix bundle, consistent with the NOE pattern observed in both yTFIIS131–309 (this work) and yTFIIS131–240 (3).

Since the mutants in Fig. 6 are also functionally defective (35) they reveal important information on the domains of yTFIIS that are required for activity. Clearly a structurally intact 3-helix bundle and zinc ribbon are required, implying that specific surfaces of these motifs are involved in interactions of yTFIIS within the RNA polymerase II elongation complex. As shown in the accompanying paper (35), inactivating mutations in these two domains that do not disrupt the structure of the protein can reveal surfaces of yTFIIS involved in specific interactions. Since the linker region is not a stable globular domain, the inactivating mutations in this region must be interpreted differently. Inactivating mutations in this domain may destabilize a conformation adopted by yTFIIS in the elongation complex or they may be involved in direct interactions with the complex. Further data is needed in order to distinguish these two possibilities.
DISCUSSION

We have used NMR spectroscopy to study the structure-function relationship of the minimal transcriptionally active portion of yeast TFIIS which spans the proteolytically defined domains II and III. Our results confirm the presence in yTFIIS_{131–309} of the 3-helix bundle and zinc ribbon structures determined previously for smaller constructs of the yeast and human proteins, respectively. NMR data for wild-type and mutant proteins indicates that the 3-helix bundle and zinc ribbon domains are structurally independent modules linked by the C-terminal portion of domain II and the N-terminal region of domain III. These intervening residues do not have a defined tertiary structure and only a small amount of secondary structure, namely two “nascent” helices between residues 214 and 235. Although the unstructured nature of the linker region makes it impossible to determine a single structure for yTFIIS_{131–309}, we summarize our structural findings with the schematic shown in Fig. 7. The relative orientation of the 3-helix bundle and the zinc ribbon are arbitrary in this view.

As described in detail in the accompanying paper (35), the 3-helix bundle and the first of the two C-terminal nascent helices of domain II comprise the RNA polymerase II-binding domain and the zinc ribbon is involved in the process of transcript cleavage and eventual read-through. Here we address the possible role of the linker region. The fact that domains II and III are not transcriptionally active either individually or in combination means that the linker region fulfills an important role that is necessary for activity. Indeed, the linker region is just as susceptible to inactivating mutations as the 3-helix bundle or the zinc ribbon. One possible role for the linker is in establishing a defined spatial relationship between the 3-helix bundle and zinc ribbon regions within the transcription elongation complex by adopting a defined conformation in the transcription complex. The linker may also participate directly in the intermolecular interactions involved in transcriptional elongation with or without adopting a folded tertiary structure. The fact that point mutants as well as insertion and deletion mutants in this region are defective for activity (Table I and Ref. 35) without changing the conformation of unbound yTFIIS suggests that a specific conformation of the linker region is involved. Such a conformation may be induced by the complex and may require an initial degree of flexibility in order to fit into place in the ternary elongation complex. A mechanism of this kind could explain the “unfolded” nature of the linker region in the absence of the elongation complex.

The structural data presented here for yTFIIS_{131–309} establishes the framework for interpretation of inactivating mutations as discussed in the accompanying paper (35). Of primary importance in this respect is our ability to distinguish between mutations that disrupt the three-dimensional fold of an important functional domain and mutations that maintain the structural integrity of the protein and therefore must be involved in functional interactions with the transcriptional machinery.

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