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Dissection of transcription factor TFIIF functional domains required for initiation and elongation

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ABSTRACT **TFIIF is unique among the general transcription factors because of its ability to control the activity of RNA polymerase II at both the initiation and elongation stages of transcription. Mammalian TFIIF, a heterodimer of ≈30-kDa (RAP30) and ≈70-kDa (RAP74) subunits, assists TFIIB in recruiting RNA polymerase II into the preinitiation complex and activates the overall rate of RNA chain elongation by suppressing transient pausing by polymerase at many sites on DNA templates. A major objective of efforts to understand how TFIIF regulates transcription has been to establish the relationship between its initiation and elongation activities. Here we establish this relationship by demonstrating that TFIIF transcriptional activities are mediated by separable functional domains. To accomplish this, we sought and identified distinct classes of RAP30 mutations that selectively block TFIIF activity in transcription initiation and elongation. We propose that (*i*) TFIIF initiation activity is mediated at least in part by RAP30 C-terminal sequences that include a cryptic DNA-binding domain similar to conserved region 4 of bacterial σ factors and (*ii*) TFIIF elongation activity is mediated in part by RAP30 sequences located immediately upstream of the C terminus in a region proposed to bind RNA polymerase II and by additional sequences located in the RAP30 N terminus.**

At least eight general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, SII, and SIII) control the activity of RNA polymerase (pol) II during initiation (1) and elongation (2, 3) of eukaryotic mRNA. Among these transcription factors, only TFIIF (also referred to as factor g from *Saccharomyces cerevisiae*, factor 5 from *Drosophila melanogaster*, βγ from rat, and RAP30/74 or FC from human cells) functions in both initiation and elongation. In higher eukaryotes TFIIF is a heterodimer of ≈30-kDa (RAP30) and ≈70-kDa (RAP74) subunits (1). Both RAP30 and RAP74 play important roles in assembly of functional preinitiation complexes and in initiation and elongation (4–6). In *S. cerevisiae* TFIIF is a heterotrimer of 105-, 54-, and 30-kDa subunits; the two large subunits are homologs of RAP74 and RAP30 (7). *S. cerevisiae* TFIIF is an integral component of a pol II holoenzyme that supports activated transcription (8, 9).

How TFIIF functions in transcription initiation and elongation has not been established. Evidence suggests that TFIIF promotes transcription initiation at least in part by assisting TFIIB to recruit pol II into the preinitiation complex (1). Although TFIIF is not essential for initiation (10–13), it appears to stabilize binding of pol II to the TFIID–promoter complex (14–16), perhaps by inducing formation of protein–DNA contacts within the preinitiation complex between the TATA box and transcriptional start site (14, 15, 17).

A role for TFIIF in transcription elongation was first demonstrated by Greenleaf and coworkers (18). Subsequent studies in several laboratories have shown that TFIIF can potently activate the catalytic rate of transcribing pol II by a mechanism involving suppression of transient pausing by the polymerase at many sites on DNA templates (4, 5, 19–21). In this respect, TFIIF functions similarly to general elongation factor SIII (21, 22), but differently than elongation factor SII (2, 3, 23), which promotes release of pol II from pauses induced by a variety of transcriptional impediments, including DNA-bound proteins (24) and drugs (25) and specific attenuation sites found in such genes as the histone H3.3 (26, 27), adenovirus 2 major late (AdML) (28, 29), and adenosine deaminase (30) genes.

A major objective of efforts to understand how TFIIF controls transcription by pol II is to establish the relationship between its initiation and elongation activities. Efforts to define TFIIF functional domains have led to the identification of potential pol II- and DNA-binding domains in the RAP30 subunit. Sopta *et al.* (31) identified a RAP30 region exhibiting significant sequence similarity with the pol-binding domains of *Escherichia coli* σ^{70} and *Bacillus subtilis* σ^{43}. McCracken and Greenblatt (32) subsequently observed that protein kinase A-catalyzed phosphorylation of a serine residue at the C terminus of this RAP30 region was blocked when TFIIF and pol II were mixed, suggesting that TFIIF may indeed interact with pol II through this RAP30 region. Garrett *et al.* (33) identified a RAP30 C-terminal region exhibiting significant sequence similarity with the highly conserved DNA-binding domain present in C-terminal region 4 of bacterial σ factors. Tan *et al.* (34) subsequently demonstrated that the C terminus of RAP30 can bind DNA and, moreover, that there is a close correlation between the DNA-binding and transcription activities of RAP30 C-terminal deletion mutants, suggesting that TFIIF may stabilize the preinitiation complex by binding directly to promoter DNA through its RAP30 subunit. This model is consistent with the results of Coulombe *et al.* (17), who found that RAP30 could be crosslinked in the preinitiation complex to promoter sequences between the TATA box and the transcription start site.

To establish the relationship between TFIIF initiation and elongation activities and to delimit TFIIF functional domains, we have now carried out a more systematic structure/function analysis of the TFIIF RAP30 subunit. This study led to the identification of three distinct classes of RAP30 mutants: mutants defective in initiation, mutants defective in elongation, and one mutant defective in both initiation and elongation and unable to bind stably to RAP74. Here we present these findings, which demonstrate that TFIIF initiation and elongation activities are mediated by separable functional domains.

MATERIALS AND METHODS

Construction of RAP30 Internal Deletion Mutants. RAP30 internal deletion mutants were constructed by oligonucleotide-directed mutagenesis (35) of M13mpET-HRAP30 (36)

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Abbreviations: AdML, adenovirus 2 major late; His-, histidine-tagged; pol, RNA polymerase; TBP, TATA-binding protein.

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with the Muta-Gene M13 *in vitro* mutagenesis kit (Bio-Rad) and confirmed by dideoxy DNA sequencing with Sequenase version 2.0 (United States Biochemical). Mutagenic oligonucleotides included 15 nt from the parent rat RAP30 sequence (33) on either side of the deletion point.

Expression and Purification of TFIIF Mutants. Intact TFIIF heterodimers containing wild-type or mutant RAP30 and RAP74 were produced by coexpression of histidine-tagged (His-) RAP30s and untagged RAP74 in *E. coli* JM109(DE3) as described (36), except that *E. coli* cultures were 1 liter and bacterial cells were infected with M13mpET-RAP74 at a multiplicity of ≈100 and with M13mpET-HRAP30 and derivatives at a multiplicity of ≈10. TFIIF was purified from supernatants of bacterial cell lysates by Ni^{2+}-nitrilotriacetic acid-agarose (Invitrogen) affinity chromatography, and contaminating free RAP30 was removed by phosphocellulose (P11; Whatman) chromatography (37). The resulting TFIIF-containing fractions were dialyzed against buffer [40 mM Tris·HCl, pH 7.5, 0.5 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol] containing 100 mM KCl, applied to a TSK DEAE-NPR column (35 mm × 4.6 mm; Toso-Haas) on a SMART system (Pharmacia Biotech, Piscataway, NJ), and eluted with a 3-ml linear gradient of 100–400 mM KCl in the same buffer. Wild-type and mutant TFIIFs were eluted with ≈250 mM KCl.

RESULTS

Expression of TFIIF Mutants and Identification of RAP30 Sequences Needed for Interaction with RAP74. To establish the relationship between TFIIF initiation and elongation activities, TFIIF mutants with short internal deletions in their RAP30 subunits (Fig. 1) were constructed, expressed in *E. coli*, and tested for their abilities (*i*) to support synthesis of correctly initiated runoff transcripts from the AdML promoter in a TFIIF-dependent basal transcription system reconstituted with purified pol II, TATA-binding protein (TBP), TFIIB, TFIIE, and TFIIH and (*ii*) to stimulate the rate of RNA chain elongation by pol II using an oligo(dC)-tailed template.

Because individually expressed and purified RAP30 and RAP74 do not assemble efficiently *in vitro* to reconstitute TFIIF heterodimers (43) that are active in transcription elongation (5), we have developed an expression strategy (36) involving coinfection of *E. coli* with recombinant M13 bacteriophage vectors carrying cDNAs encoding wild-type or mutant His-RAP30s and untagged RAP74. Under these conditions, wild-type RAP30 assembles with RAP74 to form a stable heterodimer that can be readily isolated from supernatants of bacterial lysates by affinity chromatography on Ni^{2+}-nitrilotriacetic acid-agarose (Fig. 2). Using this coexpression strategy, we screened RAP30 deletion mutants for their abilities to assemble with RAP74 to form stable heterodimers.

As shown by Western blots (Fig. 3), all of the mutant His-RAP30s except Δ16–30 were able to assemble with RAP74 to form mutant TFIIF heterodimers that could be isolated by Ni^{2+} affinity chromatography; unlike all other mutants tested, Δ16–30 did not retain RAP74 on Ni^{2+}-agarose, even though this mutant binds efficiently to the resin. The Δ16–30 deletion falls within a 110-aa N-terminal RAP30

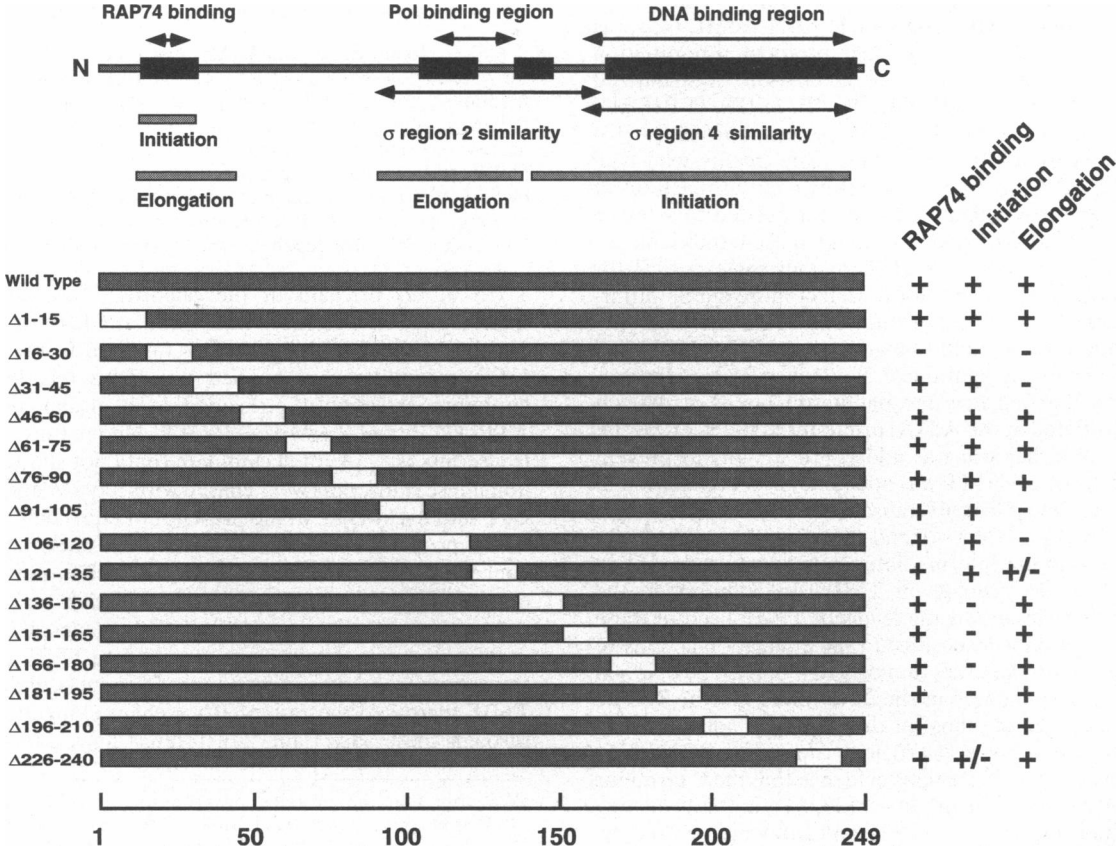

FIG. 1. Summary of RAP30 mutants and their functional properties. At left, structures of RAP30 mutants are represented with deleted residues indicated. At right, the results of assays described in the text and shown in Figs. 3 and 4 are summarized. In the diagram at the top, black boxes indicate regions identified by the MACAW program (38) as most highly conserved among RAP30s from human (31, 39), rat (33, 40), *X. laevis* (41), *D. melanogaster* (GenBank accession no. U02461), and *S. cerevisiae* (7); a region 2 similarity, RAP30 region proposed to be related to region 2 of *E. coli* σ^{70} and *B. subtilis* σ^{43} (31); Pol binding region, RAP30 region proposed (32) to be similar to the *E. coli* σ^{70} pol-binding region (42); σ region 4 similarity, RAP30 region proposed to be related to region 4 of bacterial σ factors (33); DNA binding region, RAP30 region shown to be capable of binding DNA (34). RAP30 regions shown in this study to be needed for interaction with RAP74 and to be most critical for TFIIF initiation and elongation activities are also indicated.

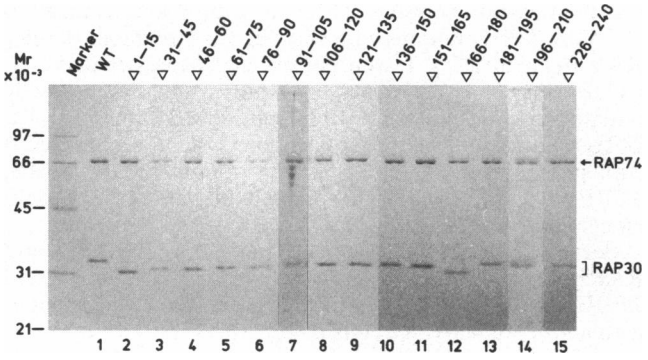

FIG. 2. SDS/PAGE analysis of purified TFIIF mutants. Samples (≈0.5 μg) of Ni^{2+}-agarose-purified wild-type (WT) TFIIF and TFIIF mutants were analyzed by SDS/10% polyacrylamide gel electrophoresis. Protein was visualized by staining gels with Coomassie brilliant blue. The mutant Δ196–210 protein used was the phosphocellulose fraction; all other TFIIF derivatives were purified through the DEAE-NPR step.

region required for interaction with RAP74 (44). The Δ16–30 mutant lacks a short N-terminal region that is rich in hydrophobic amino acids and that is highly conserved among RAP30s from yeast to mammals (Fig. 4). This conserved hydrophobic patch may constitute a surface for interaction with RAP74.

Identification of RAP30 Mutations That Disrupt TFIIF Initiation and Elongation Activities. To measure the activity of TFIIF mutants in promoter-specific initiation, runoff transcription from the AdML promoter in pDN-AdML (45) was carried out by a heparin challenge protocol. Preinitiation complexes were assembled by preincubation of linearized pDN-AdML DNA with pol II, TBP, TFIIB, TFIIE, TFIIH, and either wild-type TFIIF, TFIIF mutants, or a mixture of RAP74 and either RAP30 or Δ16–30. In these experiments, wild-type and mutant TFIIFs or RAP30s were included in reactions at a concentration approximately twice that needed to saturate the assay with wild-type protein; under these conditions, we expected to identify those RAP30 regions most critical for TFIIF initiation activity but not to detect more subtle differences between the activities of different TFIIF mutants.

Transcription was initiated by addition of ATP, UTP, and [α-^{32}P]CTP. This combination of NTPs is sufficient for synthesis of only the first few phosphodiester bonds of nascent transcripts initiated at the AdML promoter in pDN-AdML (5, 45). After 3 min, heparin was added (10 μg/ml) to prevent further initiations, and GTP was added to allow completion of synthesis of full-length runoff transcripts. As shown previously (5, 46) and in Fig. 5 (lanes 1 and 2), formation of heparin-resistant transcription intermediates (and hence transcription initiation) depends strongly on TFIIF. In addition, in the presence of heparin and at the levels of TFIIF used in these assays, transcription is insensitive to the elongation activity of TFIIF (5, 21). With the exception of Δ16–30, RAP30 deletion mutants lacking sequences in the N-terminal half of RAP30 [aa 1–135 and including most of the proposed pol II-binding domain (31, 32)] supported efficient transcription initiation (Fig. 5). Consistent with previous evidence that the C terminus of RAP30 is required for initiation (33, 34), mutants lacking RAP30 sequences between aa 136 and 210 were profoundly impaired in their ability to support transcription initiation (Fig. 5, lanes 11–15), and the mutant Δ226–240 was partially impaired (lane 16); as shown below, however, each of these mutants stimulated elongation.

The elongation activity of TFIIF mutants was assessed with the tailed-template assay (47) using the oligo(dC)-tailed plasmid pCpGR220 S/P/X (48) as template. As diagrammed in Fig. 5 *Lower*, the first nontemplate-strand dT residues are

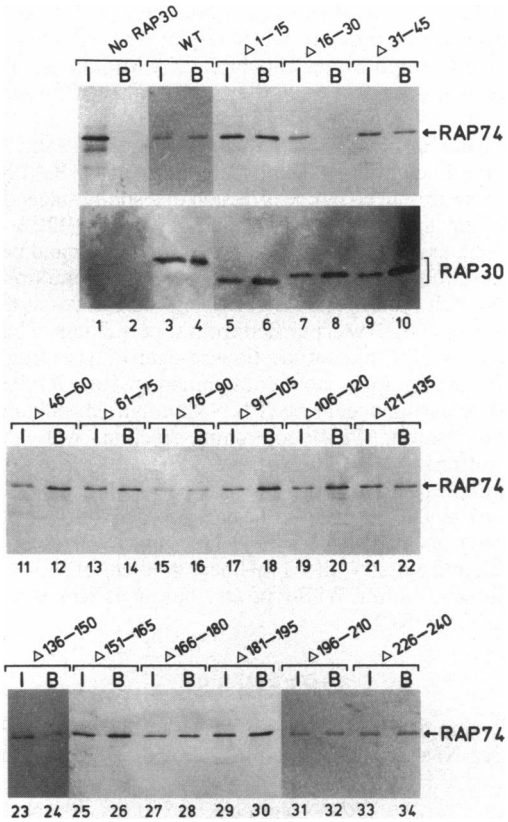

FIG. 3. Identification of a highly conserved RAP30 region needed for interaction with RAP74. Lysates (input fractions, I) from M13mpET-infected cells expressing RAP74 alone or RAP74 and either wild-type (WT) or mutant His-RAP30s were applied to Ni^{2+}-agarose resin (Invitrogen) and eluted (bound fractions, B) as described (36). Equal volumes of the input and bound fractions were analyzed by SDS/10% polyacrylamide gel electrophoresis followed by Western blotting using anti-RAP74 (generous gift of S. Kitajima, Kyushu University, Fukuoka, Japan) or anti-RAP30 antibodies as probes.

≈135 nt downstream of the oligo(dC) tail, and the next nontemplate dT residues are ≈250 nt downstream of the oligo(dC) tail. Transcription was initiated in the absence of UTP by addition of purified pol II to reaction mixtures containing the oligo(dC)-tailed pGR220 plasmid template and ATP, GTP, and 2 μM [α-^{32}P]CTP. Under these conditions, transcripts of ≈135 nt accumulate (data not shown). After 15 min, these transcripts were chased with 100 μM nonradioactive CTP and 2 μM UTP in the presence of equivalent amounts of wild-type TFIIF or TFIIF mutants.§ At these limiting UTP concentrations and in the absence of TFIIF, the majority of elongating pol II stalled within the dT-rich region ≈250 nt downstream of the oligo(dC) tail (Fig. 5, lane 19). As shown previously (5), in the presence of wild-type TFIIF the ≈250-nt transcripts were chased into longer products, indicating that TFIIF increased the rate of RNA chain elongation by pol II through these sites (lane 20). Interestingly, Δ16–30 was the

§We note that the protein concentration of wild-type or mutant TFIIFs in reaction mixtures is an amount that does not fully saturate the elongation assay with wild-type TFIIF. Titration of TFIIF to levels much greater than those required to saturate the transcription initiation assay still produces a dose–response in the oligo(dC)-tailed-template assay for TFIIF elongation activity (ref. 5, figure 3). As we showed previously (ref. 21, figure 10), the apparent specific activity of wild-type TFIIF in initiation is greater than its apparent specific activity in elongation, consistent with the model, first proposed by Price *et al.* (18), that although TFIIF appears to function stoichiometrically with components of the preinitiation complex, it is in a dynamic equilibrium with the elongation complex.

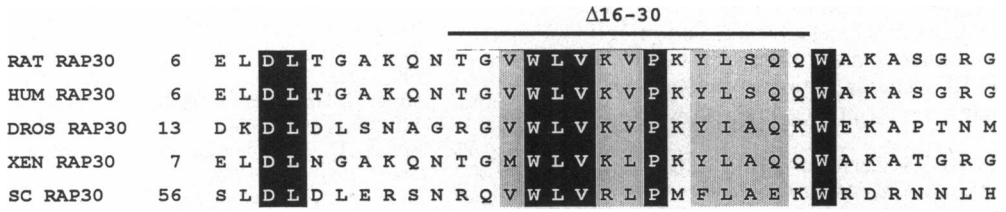

FIG. 4. Multiple sequence alignment of RAP30 region needed for interaction with RAP74. RAP30 sequences from human (HUM) (31, 39), rat (33, 40), *X. laevis* (XEN) (41), *D. melanogaster* (DROS) (GenBank accession no. U02461), and *S. cerevisiae* (SC) (7) were compared. Black boxes indicate identical amino acids. Gray boxes indicate similar amino acids.

only mutant tested that was severely impaired in both initiation (lanes 17 and 18) and elongation (lanes 35 and 36). In addition, whereas RAP30 mutants (with the exception of Δ16–30) that lack portions of the RAP30 N terminus through aa 135 supported transcription initiation, RAP30 mutant Δ31–45, which lacks residues immediately C-terminal to the region needed for interaction with RAP74, and RAP30 mutants Δ91–105, Δ106–120, and Δ121–135, which span the proposed pol II-binding domain (31, 32), were almost completely inactive in elongation (lanes 22 and 26–28). Thus, the TFIIF initiation and elongation activities can be functionally resolved by mutations in the RAP30 subunit.

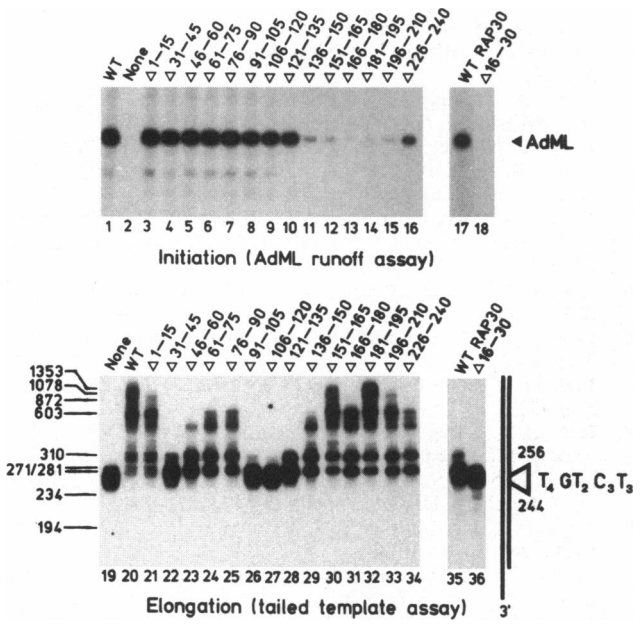

FIG. 5. TFIIF initiation and elongation activities are mediated by separable functional domains. Runoff transcription reactions (lanes 1–18) were performed (5) with a heparin challenge protocol. Reaction mixtures contained 100 ng of *Nde* I-digested pDN-AdML DNA (45) and ≈10 ng of recombinant TFIIB, 10 ng of recombinant TFIIE, 40 ng of TFIIH (phenyl fraction), 50 ng of recombinant rat TBP, 0.01 unit of pol II, and either 10 ng of wild-type TFIIF (WT, lane 1), 10 ng of TFIIF mutant (lanes 3–16), or a combination of 10 ng of RAP74 and 5 ng of RAP30 (lane 17) or Δ16–30 (lane 18). NTPs were present in reaction mixtures at final concentrations of 50 μM ATP, 50 μM UTP, 50 μM GTP, 10 μM CTP, and 8 μCi of [α-^{32}P]CTP (600 Ci/mmol; Amersham; 1 Ci = 37 GBq). Oligo dC-tailed-template reactions (lanes 19–36) were performed as described (5) with 100 ng of oligo(dC)-tailed pGR220 template (48), 0.01 unit of pol II, and 100 ng of wild-type TFIIF (lane 20) or TFIIF mutants (lanes 21–34) or a combination of 100 ng of RAP74 and 50 ng of RAP30 (lane 35) or Δ16–30 (lane 36). During the pulse-labeling phase of the assay, NTPs were present in reaction mixtures at concentrations of 50 μM ATP, 50 μM GTP, 2 μM CTP, and 5 μCi of [α-^{32}P]CTP. An additional 2 μM UTP and 100 μM nonradioactive CTP were added to reaction mixtures during the chase phase of the assay.

DISCUSSION

Since the discovery that TFIIF controls the activity of pol II at both the initiation and the elongation stage of transcription, a major goal of research on TFIIF has been to establish the relationship between its initiation and elongation activities. In this report, we demonstrate that TFIIF transcriptional activities are mediated by separable functional domains. To accomplish this, we sought and identified distinct classes of RAP30 mutations that selectively blocked TFIIF activity in transcription initiation and elongation. Characterization of these RAP30 mutants has revealed that TFIIF transcriptional activities are governed by several distinct RAP30 regions.

Of all RAP30 mutants tested, only one, Δ16–30, was inactive in both transcription initiation and elongation. This was also the only mutant unable to form stable heterodimers with RAP74. Because RAP74 is required in our assays for transcription initiation from the AdML promoter and for elongation (5), this finding suggests (*i*) that interaction of RAP30 and RAP74 is essential for both TFIIF initiation and elongation activities, (*ii*) that this RAP30 region has more than one function in transcription, or (*iii*) that deletion of this region affects the overall tertiary structure of RAP30. Disruption of two separate RAP30 regions rendered TFIIF inactive in transcription elongation without abrogating its ability to support initiation. The RAP30 mutant Δ31–45, which lacks the sequence immediately C-terminal to the region needed for interaction with RAP74, was capable of supporting efficient initiation but was impaired in elongation. In addition, RAP30 mutants lacking sequences that fall between residues 91 and 135 and that overlap most of the proposed pol II-binding domain (32) could support initiation but were severely impaired in transcription elongation. Finally, RAP30 mutants lacking sequences that fall between residues 136 and 210 and that include a region capable of binding DNA (34) were active in elongation but severely impaired in initiation.

The tailed-template assays used to measure TFIIF elongation activity provide a measure of the interaction of TFIIF with the pol II elongation complex, since the assay mixtures contain no other transcription factors. The deleterious effect of mutations between RAP30 residues 91 and 135 on TFIIF elongation activity is therefore consistent with the proposal (32) that this region contains a bona fide pol II-binding domain. That the initiation function of TFIIF does not require the presence of the proposed RAP30 pol II-binding domain was unexpected but not necessarily surprising. During initiation, RAP30 is likely to interact directly or indirectly through RAP74 with multiple components of the preinitiation complex, including template DNA (17, 34), TFIIB (49), and TFIIE and/or TFIIH (50–52); the combination of these interactions could provide sufficient binding energy for stable association of TFIIF with preinitiation intermediates.

The coevolution of distinct initiation and elongation activities in a single protein makes TFIIF unique among previously described transcription factors involved in synthesis of both prokaryotic and eukaryotic mRNA. The availability of distinct classes of TFIIF mutants defective in transcription initiation

and elongation will provide a powerful tool for defining in greater detail the biochemical mechanism(s) governing TFIIF action and should ultimately allow dissection of the relative contributions of TFIIF to the initiation and elongation stages of basal and activated transcription in eukaryotes.

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