Characterization of Elongin C Functional Domains Required for Interaction with Elongin B and Activation of Elongin A*

Yuichiro Takagi‡§, Ronald C. Conaway‡, and Joan Weliky Conaway‡§¶

From the §Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104 and ¶Department of Biochemistry and Molecular Biology, the University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

The Elongin (SIII) complex stimulates the rate of elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites along DNA templates. The Elongin (SIII) complex is composed of a transcriptionally active A subunit, a chaperone-like B subunit, which promotes assembly and enhances stability of the Elongin (SIII) complex, and a regulatory C subunit, which (i) functions as a potent activator of Elongin A transcriptional activity, (ii) interacts specifically with Elongin B to form an isolable Elongin BC complex, and (iii) is bound and negatively regulated in vitro by the product of the von Hippel-Lindau tumor suppressor gene. As part of our effort to understand how Elongin C regulates the activity of the Elongin (SIII), we are characterizing Elongin C functional domains. In this report, we identify Elongin C mutants that fall into multiple functional classes based on their abilities to bind Elongin B and to bind and activate Elongin A under our assay conditions. Characterization of these mutants suggests that Elongin C is composed of multiple overlapping regions that mediate functional interactions with Elongin A and B.

Eukaryotic messenger RNA synthesis is an elaborate biochemical process catalyzed by multisubunit RNA polymerase II and governed by the concerted action of a set of general transcription factors that control the activity of polymerase during the initiation and elongation stages of transcription (1–4). At least six general initiation factors (TFIIF, TFIID, TFIIA, TFIIE, TFIIF, and TFIH) have been identified in eukaryotic cells and found to promote selective binding of RNA polymerase II to promoters and to support a basal level of transcription (1). In addition to the general initiation factors, five general elongation factors (TFIIB, SII, TFIIF, ELL, and Elongin (SIII)) have been defined biochemically and found to increase the efficiency of elongation by RNA polymerase II. P-TEFb catalyzes the conversion of early, termination-prone elongation complexes into productive elongation complexes (5, 6). SII prevents RNA polymerase II from terminating transcription prematurely by promoting passage of polymerase through a variety of transcriptional impediments, including DNA sequences that act as intrinsic arrest sites and DNA-bound proteins and drugs (7). The remaining elongation factors, TFIIF (8), ELL (9), and Elongin (SIII) (10, 11) all act to increase the overall rate of elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites along the DNA. In addition, two of these elongation factors, ELL and Elongin (SIII), may play roles in the development of certain types of cancers. The gene encoding ELL is a frequent target for t(11;19) chromosomal translocations in acute myeloid leukemias (12, 13). Elongin (SIII) is a potential target for regulation by the product of the von Hippel-Lindau (VHL) tumor suppressor gene, which is mutated in the majority of clear-cell renal carcinomas and in families with VHL disease, a rare genetic disorder that predisposes individuals to a variety of cancers including clear-cell renal carcinoma, hemangioblastomas and hemangiomas, and pheochromocytomas (14, 15).

Elongin (SIII) was originally purified from mammalian cells as a heterotrimer composed of A, B, and C subunits of 773, 118, and 112 amino acids, respectively (10, 16–18). Elongin A is the transcriptionally active component of the Elongin (SIII) complex. Whereas Elongin A is capable of weakly stimulating the rate of elongation by RNA polymerase II in the absence of Elongin B and C, neither Elongin B nor Elongin C affects the activity of polymerase in the absence of Elongin A (16–18). Biochemical studies have shown that Elongin B and C are positive regulators of Elongin A activity and function by different mechanisms (16–18). Elongin C is capable of interacting directly with Elongin A in the absence of Elongin B to form an AC complex with increased specific activity, suggesting that Elongin C functions as a direct activator of Elongin A. Elongin B, a member of the ubiquitin homology gene family, does not appear to interact directly with Elongin A. Evidence suggests that Elongin B plays a chaperone-like role in assembly of the Elongin (SIII) complex by binding to Elongin C and facilitating its interaction with Elongin A.

As part of our effort to understand the structure, mechanism of action, and regulation of the Elongin (SIII) complex, we are carrying out a systematic structure-function analysis of the each of the Elongin subunits. Here we describe studies leading to the identification of Elongin C regions important for binding to Elongin B and for binding and activation of Elongin A.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled ultrapure ribonucleoside 5′-triphosphates were purchased from Pharmacia Biotech Inc. Restriction enzymes were...
Elongin C Functional Domains

ml of 5.7 μm guanidine hydrochloride, 40 mM Tris-HCl (pH 7.9), 40 mM imidazole (pH 8.0), and 0.5 mM PMSF, and 0.5 μM RNasin and recombinant Elongin A was eluted with 4.2 μm guanidine hydrochloride, 40 mM Tris-HCl (pH 7.9), 300 mM imidazole (pH 8.0), and 0.5 mM PMSF.

Overexpression of Elongin B and wild type and mutant Elongin C was accomplished using the M13mpET bacteriophage expression system (16, 17). A 100-μl culture of E. coli strain JM109(DE3) (Promega) was grown to an A600 of 0.6 in LB medium at 37°C. Cells were infected with M13mpET bacteriophage at 50 moi for the wild type or mutant rat Elongin C DNAs at a multiplicity of infection of 10–20. After an additional 2 h at 37°C, cells were induced with 0.5 mM IPTG, and cultures were incubated an additional 3 h. Cells were harvested by centrifugation at 2000 × g for 10 min at 4°C. The cell pellet was suspended in 7 ml of 20 mM Tris-HCl (pH 8.0), 10 mM imidazole (pH 8.0), and 0.5 mM PMSF, and recombinant Elongin A was purified as described above.

Assay of Elongin BC Complex Formation—6 μg of Elongin B were mixed with 6 μg of either wild type or mutant Elongin C and diluted 5-fold with 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 2 mM DTT, 50 μM ZnSO4, 0.1 mM EDTA, and 10% (v/v) glycerol. After incubation for 90 min on ice, the mixtures were dialyzed at 4°C overnight against 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 10% (v/v) glycerol. Following dialysis, the mixtures were centrifuged at 100,000 × g for 35 min. The resulting supernatants were applied to TSK DEAE-NPR columns (35 mm × 46 mm, Tosoh-Haas) pre-equilibrated in 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 40 mM KCl and fractionated using a SMART microchromatography system (Pharmacia) at 8°C. The columns were eluted at 0.3 ml/min with a 3-ml linear gradient from 0.04 to 0.5 M KCl in 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol. Aliquots of each column fraction were analyzed by 10% Tris-Tricine SDS-polyacrylamide gel electrophoresis (28), and the proteins were visualized by silver staining.

Assay of Elongin ABC Complex Formation—45 μg of Elongin A, −6 μg of Elongin B, and −6 μg of either wild type or mutant Elongin C were diluted 5-fold with 40 mM Hepes-NaOH (pH 7.9), 100 mM KCl, 50 μM ZnSO4, and 10% (v/v) glycerol. After incubation for 90 min on ice, the mixtures were dialyzed at 4°C overnight against 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 10% (v/v) glycerol, and 40 mM KCl. Following dialysis, the mixtures were centrifuged at 60,000 × g for 15 min at 4°C. The resulting supernatants were applied to TSK SP-NPR columns (35 mm × 46 mm, Tosoh-Haas) pre-equilibrated in 40 mM Hepes-NaOH (pH 7.9), 1 mM DTT, 10% (v/v) glycerol, and 0.1 M KCl and fractionated using a SMART microchromatography system (Pharmacia) at 8°C. The columns were eluted at 0.3 ml/min with a 9-ml linear gradient from 0.1 to 0.8 M KCl in 40 mM Hepes-NaOH (pH 7.9), 1 mM DTT, and 10% (v/v) glycerol. Aliquots of each column fraction were analyzed by 10% Tris-Tricine SDS-polyacrylamide gel electrophoresis (28), and the proteins were visualized by silver staining.

RESULTS

Identification of an Elongin C Region Important for Binding to Elongin B—Mammalian Elongin C is a 112-amino acid protein with a calculated molecular mass of 12,473 Da (16). In previous studies, we have shown that transcriptionally active Elongin (SIII) and Elongin subassemblies can be reconstituted by reconstitution of denatured native Elongin subunits purified from rat liver (10, 16) or bacterially expressed Elongin subunits purified from guanidine hydrochloride-solubilized inclusion bodies (14, 16–18). To investigate the requirement for interaction of Elongin C with Elongin B, a systematic series of N-terminal, C-terminal, and internal Elongin C deletion mutants were constructed (Fig. 1), expressed in E. coli, purified from inclusion bodies, and assayed for their abilities to form chromatographically isolable Elongin BC complexes. In these studies, individual Elongin C mutants were expressed with wild type rat Elongin A and together with wild type rat Elongin B diluted 5-fold to 0.6 mg of 5.7 μm guanidine hydrochloride, 40 mM Tris-HCl (pH 7.9), 40 mM imidazole (pH 8.0), and 0.5 mM PMSF. The column was washed with 10

Preparation of RNA Polymerase II and Transcription Factors—RNA polymerase II (21) and TFIIH (22) were purified as described from rat liver nuclear extracts. Recombinant yeast TBP (23) and rat TFIIH (24) were expressed in E. coli and purified as described. Recombinant TFIIH was prepared as described previously (25), except that the 56-kDa subunit was expressed in BL21(DE3) (pH 7.9, 0.1 mM EDTA, and 10% (v/v) glycerol). Following dialysis, the mixtures were centrifuged at 100,000 × g for 35 min. The resulting supernatants were applied to TSK DEAE-NPR columns (35 mm × 46 mm, Tosoh-Haas) pre-equilibrated in 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 40 mM KCl and fractionated using a SMART microchromatography system (Pharmacia) at 8°C. The columns were eluted at 0.3 ml/min with a 3-ml linear gradient from 0.04 to 0.5 M KCl in 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol. Aliquots of each column fraction were analyzed by 10% Tris-Tricine SDS-polyacrylamide gel electrophoresis (28), and the proteins were visualized by silver staining.

Identification of an Elongin C Region Important for Binding to Elongin B—Mammalian Elongin C is a 112-amino acid protein with a calculated molecular mass of 12,473 Da (16). In previous studies, we have shown that transcriptionally active Elongin (SIII) and Elongin subassemblies can be reconstituted by reconstitution of denatured native Elongin subunits purified from rat liver (10, 16) or bacterially expressed Elongin subunits purified from guanidine hydrochloride-solubilized inclusion bodies (14, 16–18). To investigate the requirement for interaction of Elongin C with Elongin B, a systematic series of N-terminal, C-terminal, and internal Elongin C deletion mutants were constructed (Fig. 1), expressed in E. coli, purified from inclusion bodies, and assayed for their abilities to form chromatographically isolable Elongin BC complexes. In these studies, individual Elongin C mutants were expressed with wild type rat Elongin A and together with wild type rat Elongin B diluted 5-fold to 0.6 mg of 5.7 μm guanidine hydrochloride, 40 mM Tris-HCl (pH 7.9), 40 mM imidazole (pH 8.0), and 0.5 mM PMSF. The column was washed with 10

Preparation of RNA Polymerase II and Transcription Factors—RNA polymerase II (21) and TFIIH (22) were purified as described from rat liver nuclear extracts. Recombinant yeast TBP (23) and rat TFIIH (24) were expressed in E. coli and purified as described. Recombinant TFIIH was prepared as described previously (25), except that the 56-kDa subunit was expressed in BL21(DE3) (pH 7.9, 0.1 mM EDTA, and 10% (v/v) glycerol). Following dialysis, the mixtures were centrifuged at 100,000 × g for 35 min. The resulting supernatants were applied to TSK DEAE-NPR columns (35 mm × 46 mm, Tosoh-Haas) pre-equilibrated in 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 40 mM KCl and fractionated using a SMART microchromatography system (Pharmacia) at 8°C. The columns were eluted at 0.3 ml/min with a 3-ml linear gradient from 0.04 to 0.5 M KCl in 40 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol. Aliquots of each column fraction were analyzed by 10% Tris-Tricine SDS-polyacrylamide gel electrophoresis (28), and the proteins were visualized by silver staining.
those of both wild type Elongin B, which flows through TSK DEAE-NPR at low ionic strength, and wild type Elongin C, which binds tighter to this resin than the Elongin BC complex (Fig. 2A) and elutes over a broad range of ionic strength. Thus, coelution of Elongin Band C from TSK DEAE-NPR is diagnostic of an interaction between the two proteins.

As shown in Fig. 2B, deletion of as many as 28 amino acids from the C terminus of Elongin C does not prevent formation of isolable Elongin BC complexes. Likewise, deletion of as many as 18 amino acids from the N terminus of Elongin C does not prevent formation of isolable Elongin BC complexes. Deletion of 22 amino acids from the N terminus of Elongin C, however, abolished formation of isolable Elongin BC complexes, suggesting that Elongin C residues between 18 and 22 are critical for interaction of Elongin B and C. Consistent with this possibility, an Elongin C internal deletion mutant lacking residues 21–30 did not form an isolable Elongin BC complex (Fig. 2B); this mutant was the only Elongin C internal deletion mutant that failed to bind to Elongin B, suggesting that the Elongin C region between residues 18 and 22 is critical for interaction of Elongin B and C. Consistent with this possibility, an Elongin C internal deletion mutant lacking residues 18–22 was inactive. Furthermore, Elongin BC complexes containing all Elongin C internal deletion mutants lacking sequences C-terminal to amino acid 61 were inactive, and an Elongin BC complex containing the internal deletion mutant C-(Ala21–30), which lacks sequences immediately C-terminal to the region important for Elongin B binding, was also inactive. Finally, Elongin BC complexes containing the alanine scanning mutant C-(Ala19–21) were inactive, although BC complexes containing C-(Ala22–24) was active (Fig. 2C).

Although Elongin B facilitates assembly and enhances stability of the Elongin (SIII) complex, it is not essential for activation of Elongin A by wild type Elongin C (18). As de-
ing Elongin A.

to assemble into Elongin BC complexes was capable of activating Elongin A, none of the Elongin C mutants that failed to assemble into Elongin BC complexes were capable of activating Elongin A. As shown in Fig. 5, the N-terminal Elongin C deletion mutants C-(15–112) and C-(19–112), which form isolable Elongin BC complexes that activate Elongin A, were capable of assembling into isoalable Elongin ABC complexes, whereas the remaining N-terminal and C-terminal Elongin C deletion mutants, which either fail to form Elongin BC complexes or form inactive BC complexes, were unable to form isolable Elongin ABC complexes. In contrast, with the exception of Elongin C internal deletion mutant C-(Δ21–30), which lacks residues 21–30 and does not form an isolable Elongin BC complex, each of the Elongin C internal deletion mutants was capable of forming an isolable Elongin ABC complex (Fig. 6A). In these experiments, the yield of Elongin B and C in purified Elongin ABC complexes containing Elongin C internal deletion mutants was routinely less than their yield in purified Elongin ABC complexes containing wild type Elongin C, suggesting that Elongin ABC complexes containing the internal deletion mutants assemble less efficiently or are less stable than wild type Elongin ABC.

Runoff transcription assays were used to compare the activities of the isolated wild type and mutant Elongin ABC complexes shown in Fig. 6A. The concentration of the wild type Elongin ABC complex in the reaction shown in lane 2 of Fig. 6B was sufficient to saturate the assay, and the concentrations of mutant Elongin ABC complexes were adjusted so that all reactions shown in Fig. 6B contained equivalent levels of Elongin A. Highlighting the importance of sequences at the C terminus of Elongin C for activation of Elongin A, Elongin ABC complexes containing C-(Δ91–100) did not stimulate the rate of elongation by RNA polymerase II. Likewise, Elongin ABC complexes containing C-(Δ61–70), which forms Elongin BC complexes with aberrant chromatographic properties, were inactive. Interestingly, although Elongin BC complexes containing Elongin C internal deletion mutants C-(Δ71–80) and C-(Δ81–90) were unable to activate Elongin A, Elongin ABC complexes containing these same Elongin C mutants were capable of stimulating the rate of elongation by RNA polymerase II, suggesting that the Elongin C region between amino acids 71 and 90 is not critical for activation of Elongin A in pre-assembled Elongin ABC complexes. In these experiments, it is noteworthy that the activity of mutant Elongin ABC complexes does not, in all cases, correlate with the amount of Elongin B and C present, since Elongin ABC complexes containing Elongin C internal deletion mutant C-(Δ71–80) are considerably more active than those containing C-(Δ81–90), even though Elongin ABC complexes containing C-(Δ81–90) contain more Elongin B and C.

Thus, the results of these experiments demonstrate that assembly of Elongin C into the complete Elongin ABC complex is not sufficient for activation of Elongin A, and they indicate the importance of the C terminus of Elongin C for activation of Elongin A. Further evidence supporting the importance of the C terminus of Elongin C in activation of Elongin A came from analysis of a set of clustered alanine scanning mutants in which Elongin C residues between 89 and 112 were mutated three at a time to alanines. As predicted, all C-terminal alanine scanning mutants were capable of binding to Elongin B to form

**Fig. 2. Assay of formation of Elongin BC complexes containing wild type and mutant Elongin C.** A, wild type Elongin B (upper panel (B)), wild type Elongin C (middle panel (C)), or a mixture of wild type Elongin B and C were refolded and subjected to DEAE-NPR HPLC as described under “Experimental Procedures.” Aliquots of the indicated column fractions were analyzed by SDSPAGE, and proteins were visualized by silver staining. B, N-terminal, C-terminal, and internal Elongin C deletion mutants were assayed for their abilities to form Elongin BC complexes as described under “Experimental Procedures.” C, Elongin C alanine scanning mutants were assayed for their abilities to form Elongin BC complexes as described under “Experimental Procedures.” C-(Ala19–21) co-electrophoreses with wild type Elongin B during SDS-PAGE. L, load; FT, flow-through.

To investigate whether Elongin C deletion mutants that fail to activate Elongin A are defective in their abilities to assemble into Elongin ABC complexes, Elongin C deletion mutants were assayed for their abilities to form chromatographically isolable Elongin ABC complexes. In these experiments, individual Elongin C deletion mutants were refolded together with wild type Elongin A and B and subjected to TSK SP-NPR HPLC. As described previously, Elongin A and Elongin AC and ABC complexes bind tightly to TSK SP-NPR and can all be eluted with −0.3 M KCl, whereas Elongin B and C flow through this resin at low ionic strength (17, 18). As shown in Fig. 5, the N-terminal Elongin C deletion mutants C-(15–112) and C-(19–112), which form isolable Elongin BC complexes that activate Elongin A, were capable of assembling into isolable Elongin ABC complexes, whereas the remaining N-terminal and C-terminal Elongin C deletion mutants, which either fail to form Elongin BC complexes or form inactive BC complexes, were unable to form isolable Elongin ABC complexes. In contrast, with the exception of Elongin C internal deletion mutant C-(Δ21–30), which lacks residues 21–30 and does not form an isolable Elongin BC complex, each of the Elongin C internal deletion mutants was capable of forming an isolable Elongin ABC complex (Fig. 6A). In these experiments, the yield of Elongin B and C in purified Elongin ABC complexes containing Elongin C internal deletion mutants was routinely less than their yield in purified Elongin ABC complexes containing wild type Elongin C, suggesting that Elongin ABC complexes containing the internal deletion mutants assemble less efficiently or are less stable than wild type Elongin ABC.

Runoff transcription assays were used to compare the activities of the isolated wild type and mutant Elongin ABC complexes shown in Fig. 6A. The concentration of the wild type Elongin ABC complex in the reaction shown in lane 2 of Fig. 6B was sufficient to saturate the assay, and the concentrations of mutant Elongin ABC complexes were adjusted so that all reactions shown in Fig. 6B contained equivalent levels of Elongin A. Highlighting the importance of sequences at the C terminus of Elongin C for activation of Elongin A, Elongin ABC complexes containing C-(Δ91–100) did not stimulate the rate of elongation by RNA polymerase II. Likewise, Elongin ABC complexes containing C-(Δ61–70), which forms Elongin BC complexes with aberrant chromatographic properties, were inactive. Interestingly, although Elongin BC complexes containing Elongin C internal deletion mutants C-(Δ71–80) and C-(Δ81–90) were unable to activate Elongin A, Elongin ABC complexes containing these same Elongin C mutants were capable of stimulating the rate of elongation by RNA polymerase II, suggesting that the Elongin C region between amino acids 71 and 90 is not critical for activation of Elongin A in pre-assembled Elongin ABC complexes. In these experiments, it is noteworthy that the activity of mutant Elongin ABC complexes does not, in all cases, correlate with the amount of Elongin B and C present, since Elongin ABC complexes containing Elongin C internal deletion mutant C-(Δ71–80) are considerably more active than those containing C-(Δ81–90), even though Elongin ABC complexes containing C-(Δ81–90) contain more Elongin B and C.

Thus, the results of these experiments demonstrate that assembly of Elongin C into the complete Elongin ABC complex is not sufficient for activation of Elongin A, and they indicate the importance of the C terminus of Elongin C for activation of Elongin A. Further evidence supporting the importance of the C terminus of Elongin C in activation of Elongin A came from analysis of a set of clustered alanine scanning mutants in which Elongin C residues between 89 and 112 were mutated three at a time to alanines. As predicted, all C-terminal alanine scanning mutants were capable of binding to Elongin B to form

 prescribed above, most of the Elongin C mutants containing mutations in the region between amino acids 19 and 30 were unable to form isolable Elongin BC complexes. To investigate the ability of these Elongin C mutants to activate Elongin A, they were assayed for their abilities to stimulate the rate of accumulation of runoff transcripts synthesized by RNA polymerase II from the AdML promoter in the presence of Elongin A and the general initiation factors, but in the absence of Elongin B. As shown in Fig. 4, although wild type Elongin C strongly activated Elongin A, none of the Elongin C mutants that failed to assemble into Elongin BC complexes was capable of activating Elongin A.
chromatographically isolable Elongin BC complexes (data not shown). In addition, although Elongin BC complexes containing three of these mutants, C-(Ala89–91), C-(Ala98–99), and C-(Ala108–109), were nearly as active as wild type BC complexes, the activity of BC complexes containing C-(Ala92–94), C-(Ala95–97), C-(Ala101–103), C-(Ala104–105), and C-(Ala110–112) was significantly impaired (Fig. 7).

**DISCUSSION**

In this report, we have investigated the structure and function of Elongin C, a 112-amino acid subunit of the Elongin (SIII) complex (16, 18). Elongin (SIII) was initially purified from mammalian cells as a multimeric complex composed of A, B, and C subunits (10). Biochemical studies have shown that Elongin A is the transcriptionally active subunit of Elongin (SIII) and that Elongin B and C regulate its activity by different mechanisms (16–18). By virtue of its ability to bind directly to Elongin A in the absence of Elongin B to form an AC complex with increased specific activity, Elongin C appears to function as a bona fide activator of Elongin A. Elongin B does not appear to interact with Elongin A in the absence of Elongin C. Elongin B appears to play a chaperone-like role in formation in the Elongin (SIII) complex by binding directly to Elongin C and facilitating its interaction with Elongin A.

As part of our effort to understand how Elongin C regulates the activity of the Elongin (SIII) complex, we have constructed and analyzed a systematic series of Elongin C mutants for their abilities to bind Elongin B and to bind and activate Elongin A under our assay conditions. Elongin C mutations were found to fall into several classes based on their effects on Elongin C activities (Fig. 8). First, the only Elongin C mutations that had dramatic effects on Elongin B binding fell within a short Elongin C region between amino acids 19 and 30, consistent with the possibility that sequences within this region are directly

---

**Fig. 4.** Assay of activation of Elongin A by Elongin C mutants that do not form isolable Elongin BC complexes. Runoff transcription assays were performed as described under "Experimental Procedures" according to the protocol diagrammed at the bottom of Fig. 3A. A mixture containing ~50 ng of SP-NPR-purified Elongin A and ~5 ng (1×C) or ~50 ng (10×C) of Ni²⁺-purified and refolded wild type or mutant Elongin C was preincubated on ice for 60 min and then added to reaction mixtures at -10°C.

**Fig. 3.** Assay of activation of Elongin A by wild type and mutant Elongin BC complexes. A, runoff transcription assays were performed as described under "Experimental Procedures" according to the protocol diagrammed at the bottom. A mixture containing ~50 ng of SP-NPR-purified Elongin A and ~5 ng (1× BC) or ~50 ng (10× BC) of purified wild type or mutant Elongin BC was preincubated on ice for 60 min prior to addition to reaction mixtures. Purified Elongin BC complexes were from the following DEAE-NPR fractions shown in Fig. 2: wild type, fraction 7; BC-(15–112), fraction 5; BC-(19–112), fraction 5; BC-(1–97), fraction 6; and BC-(1–83), fraction 5. Reactions shown in lanes 1 and 7 contained no Elongin BC. WT, wild type. B, runoff transcription assays were performed as described in A with Elongin BC complexes from the following DEAE-NPR fractions shown in Fig. 2: wild type, fraction 7; BC-(Δ31–40), fraction 5; BC-(Δ41–50), fraction 7; BC-(Δ51–60), fraction 5; BC-(Δ61–70), fraction 13; BC-(Δ71–80), fraction 7; BC-(Δ81–90), fraction 7; BC-(Δ91–100), fraction 5; BC-(Δ110–120), fraction 13; BC-(Δ121–130), fraction 7; and BC-(Δ131–140), fraction 5. C, runoff transcription assays were performed as described in A with Elongin BC complexes from the following DEAE-NPR fractions shown in Fig. 2: wild type BC, fraction 7; BC-(Δ19–21), fraction 7; BC-(Δ22–24), fraction 7; BC-(Δ25–27), fraction 7; BC-(Δ28–30), fraction 7; BC-(Δ31–33), fraction 7; BC-(Δ34–36), fraction 7; BC-(Δ37–39), fraction 7; BC-(Δ40–42), fraction 7; BC-(Δ43–45), fraction 7; BC-(Δ46–48), fraction 7; BC-(Δ49–51), fraction 7; BC-(Δ52–54), fraction 7; BC-(Δ55–57), fraction 7; BC-(Δ58–60), fraction 7; BC-(Δ61–63), fraction 7; BC-(Δ64–66), fraction 7; BC-(Δ67–69), fraction 7; BC-(Δ70–72), fraction 7; BC-(Δ73–75), fraction 7; BC-(Δ76–78), fraction 7; BC-(Δ79–81), fraction 7; BC-(Δ82–84), fraction 7; BC-(Δ85–87), fraction 7; BC-(Δ88–90), fraction 7; BC-(Δ91–93), fraction 7; BC-(Δ94–96), fraction 7; BC-(Δ97–99), fraction 7; BC-(Δ100–102), fraction 7; BC-(Δ103–105), fraction 7; BC-(Δ106–108), fraction 7; BC-(Δ109–111), fraction 7; and BC-(Δ112–114), fraction 7.
involved in interactions with Elongin B. Deletion mutations in this region, however, were also found to affect the ability of Elongin C to assemble into isolable Elongin ABC complexes and to activate Elongin A transcriptional activity. Thus, it is also possible that some mutations in this region disrupt the overall tertiary structure of Elongin C. Distinguishing between these possibilities must await more detailed structural studies of Elongin C and Elongin C-containing complexes.

Second, the only Elongin C mutations that had dramatic effects on formation of isolable Elongin ABC complexes and to activate Elongin A transcriptional activity. Thus, it is also possible that some mutations in this region disrupt the overall tertiary structure of Elongin C. Distinguishing between these possibilities must await more detailed structural studies of Elongin C and Elongin C-containing complexes.

Second, the only Elongin C mutations that had dramatic effects on formation of isolable Elongin ABC complexes without affecting formation of Elongin BC complexes were mutations in the extreme C terminus of Elongin C. This result, together with our finding that all Elongin C internal deletion mutants, except C-(Δ21–30), were capable of forming isolable Elongin ABC complexes, suggests that the C terminus of Elongin C plays a crucial role in assembly of the Elongin ABC complex, possibly through direct interactions with Elongin A.

Third, Elongin C mutations that affect formation of Elongin ABC complexes are only a subset of those mutations that affect activation of Elongin A, indicating that assembly of Elongin C into ABC complexes is not sufficient for activation of Elongin A. Interestingly, the size of the Elongin C region sensitive to mutations that affect activation of Elongin A was dependent on the assay used to measure activation. In one assay, which measured the ability of Elongin BC complexes to stimulate the rate of elongation by RNA polymerase II in the presence of Elongin A, Elongin C mutations that fell within the entire C-terminal half of the protein (residues 61–112) drastically reduced Elongin C activity. In contrast, in a second assay, which measured the ability of preassembled Elongin ABC complexes to stimulate the rate of elongation by RNA polymerase II, Elongin C mutations that fell between residues 71 and 90 had a significantly reduced effect on Elongin C activity, indicating that sequences within this Elongin C region are not essential for activation of Elongin A.

Finally, our previous analysis of the predicted open reading frame of the Elongin C cDNA revealed two notable features that suggested the existence of potentially important functional domains (16). First, a FASTA search of the Swiss-Prot data base revealed that Elongin C amino acids 13–75 resemble a portion of the RNA binding domain of E. coli termination protein ρ. This region of ρ is believed to be involved in allosteric coupling of the ρ RNA binding and ATPase activities (29). In light of our evidence that large portions of the ρ-like region can be deleted without affecting Elongin C activities, it is likely that the statistically significant sequence similarity between
the two proteins does not reflect a similarity of function. Second, the extreme C terminus of Elongin C is predicted by both the Chou and Fasman (30) and Garnier et al. (31) algorithms to form a short, hydrophobic α-helix that has the potential to form a coiled-coil protein-protein interaction domain (32, 33). A similar short C-terminal α-helix with potential to form a coiled-coil has been shown to play a central role in protein-protein interactions with Elongin A.

Acknowledgments—We thank T. Aso, C. Brower, A. Dvir, D. Haque, K. Garrett, S. Tan, G. Zurawski, S. Zurawski, and D. Ligget for helpful discussions, and K. Jackson of the Molecular Biology Resource Center at the Oklahoma Center for Molecular Medicine for oligonucleotide synthesis.

REFERENCES

1. Conaway, R. C., and Conaway, J. W. (1993) Annu. Rev. Biochem. 62, 161–190
2. Kane, C. M. (1994) in Transcription: Mechanisms and Regulation (Conaway, R. C., and Conaway, J. W., eds) pp. 279–296, Raven Press, New York
3. Aso, T., Conaway, J. W., and Conaway, R. C. (1995) FASEB J. 9, 1419–1428
4. Krumm, A., Meula, T., and Groudine, M. (1993) Bioessays 15, 659–665
5. Marshall, N. F., and Price, D. H. (1992) Mol. Cell. Biol. 12, 2078–2090
6. Marshall, N. F., and Price, D. H. (1995) J. Biol. Chem. 270, 12335–12338
7. Reines, D. (1994) in Transcription: Mechanisms and Regulation (Conaway, R. C., and Conaway, J. W., eds) pp. 263–278, Raven Press, New York
8. Price, D. H., Sluder, A. E., and Greenleaf, A. L. (1989) Mol. Cell. Biol. 9, 1405–1475
9. Shilatifard, A., Lane, W. S., Jackson, K. W., Conaway, R. C., and Conaway, J. W. (1996) Science 271, 1873–1876
10. Bradsher, J. N., Jackson, K. W., Conaway, R. C., and Conaway, J. W. (1993) J. Biol. Chem. 268, 25367–25393
11. Bradsher, J. N., Tan, S., McLaury, H.-J., Conaway, J. W., and Conaway, R. C. (1993) J. Biol. Chem. 268, 25584–25603
12. Thirman, M. J., Levanit, D. A., Kohayashi, H., Simon, M. C., and Rowley, J. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12110–12114
13. Mitani, K., Kanda, Y., Ogawa, S., Tanaka, T., Inazawa, J., Yazaki, Y., and Hirai, H. (1995) Blood 85, 2017–2024
14. Duan, D. R., Pause, A., Burgess, W. H., Aso, T., Chen, D. Y. T., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M., and Klausner, R. D. (1995) Science 269, 1402–1406
15. Kibel, A., Iliopoulos, O., DeCaprio, J. A., and Raelin, W. G. (1995) Science 269, 1444–1446
16. Garrett, K. P., Tan, S., Bradsher, J. N., Lane, W. S., Conaway, J. W., and Conaway, R. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5237–5241
17. Garrett, K. P., Aso, T., Bradsher, J. N., Foundlind, S. I., Lane, W. S., Conaway, R. C., and Conaway, J. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7172–7176
18. Aso, T., Lane, W. S., Conaway, J. W., and Conaway, R. C. (1995) Science 269, 1439–1443
19. Conaway, R. C., and Conaway, J. W. (1988) J. Biol. Chem. 263, 2962–2968
20. Sambrook, J., Frithch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Conaway, J. W., and Conaway, R. C. (1990) Science 248, 1550–1553
22. Conaway, J. W., Bradsher, J. N., and Conaway, R. C. (1992) J. Biol. Chem. 267, 10142–10148
23. Conaway, J. W., Hanley, J. P., Garrett, K. P., and Conaway, R. C. (1991) J. Biol. Chem. 266, 7804–7811
24. Tsuoi, A., Conger, K., Garrett, K. P., Conaway, R. C., Conaway, J. W., and Arai, N. (1992) Nucleic Acids Res. 20, 3250
25. Peterson, M. G., Inostroza, J., Maxon, M. E., Flores, O., Admon, A., Reinberg, D., and Tjian, R. (1991) Nature 354, 369–373
26. Tan, S., Conaway, R. C., and Conaway, J. W. (1994) BioTechniques 16, 824–828
27. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
28. Schagger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
29. Richardson, J. F. (1986) J. Biol. Chem. 271, 1251–1254
30. Chou, P. Y., and Fasman, G. D. (1974) Biochemistry 13, 222–228
31. Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) J. Mol. Biol. 120, 97–120
32. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988) Science 240, 1759–1764
33. O’Shea, E. K., Rutkowski, R., and Kim, P. S. (1989) Science 243, 538–542
34. Lewis, S. A., Ivanov, I. E., Lee, G.-H., and Cowan, N. J. (1989) Nature 342, 498–505