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31. We do not wish to imply that GPI-anchored proteins can never enter caveolae. Antibody–cross-linked alkaline phosphatase clusters and slowly enters caveolae for endocytosis to endosomes and lysosomes (*41*), consistent with our studies of internalization of modified albumins by caveolae, with the exception that the process of binding, clustering, internalization, and degradation was much quicker for the albumins (*19, 43*). It appears that cell surface processing, at least for GPI-linked proteins, probably comprises three distinct sequential steps: (i) induced movement of GPI-anchored proteins (probably by a ligand) into microdomains near the caveolae, thereby increasing the local concentration of GPI-linked proteins by direct sequestration of previously free molecules or possibly by assembly of several smaller clusters; (ii) eventual movement into the caveolae; and (iii) fission or budding of the caveolae from the membrane for potocytosis or endocytosis.
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33. Specialized glycolipid domains are resistant to detergent extraction and are necessary for maintaining detergent-resistant clusters of GPI-linked proteins (*2–5*). Removal of cholesterol from plasma membranes can dissociate or prevent the formation of such clusters and assure a random, free distribution of GPI-anchored proteins (*39*). As expected, cholesterol removal reduces the resistance of GPI-linked proteins to detergent solubilization (*5*), consistent with the notion that the freely diffusing GPI-anchored proteins are indeed more readily solubilized by detergents than the less mobile GPI-anchored proteins in the glycolipid domains. Moreover, in the absence of glycolipids, GPI-anchored proteins are readily solubilized from membranes by cold Triton X-100; solubility decreases with the addition of appropriate glycolipids (*2*). Thus, GPI-anchored proteins randomly distributed at the cell surface should be susceptible to detergent extraction; indeed, our percentages agree with those from the diffusion studies discussed in the text. In homogenates of non–silica-coated rat lung, ~60% of CA and 75% of 5′-NT are solubilized by Triton X-100 at 4°C. Moreover, mass balances performed on the silica-coated membranes showed that ~20% of 5′-NT and 40% of CA could be isolated in the intact, detergent-resistant membrane fraction TI.
34. As noted in (*13*), caveolae can be isolated without exposure to Triton X-100, but less efficiently. The usual protocol was followed for caveolae isolation, with the exception that Triton X-100 was omitted and, for shearing purposes, the number of homogenization strokes was increased to 48 to 60 from 12.
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45. The membrane preparations were processed for immuno–electron microscopy by a modification of a previously described procedure [P. De Camilli, S. M. Harris Jr., W. B. Huttner, P. J. Greengard, *J. Cell Biol.* **96**, 1355 (1983)]. The detergent-resistant membranes from rat lung (TI) isolated as described (*26*) directly in suspension from the sucrose gradient were mixed with 4% agarose (Seaplaque; FMC Bioproducts) in phosphate-buffered saline (PBS) at 37°C at a vesicle:agarose volume ratio of 1:3. The suspension was transferred by capillary action into a frame prepared from two glass slides separated at each end by two cover slips (0.17 mm thick). After cooling immediately to solidify the agarose, the frame was removed and the agarose cut into 2-mm squares, which were then incubated in 5% ovalbumin in PBS for 30 min, washed briefly in PBS, and incubated with anti-CA, anti-G_{M1}, or nonimmune serum overnight at 4°C. After five 10-min washes in PBS, the blocks were incubated for 8 hours at 4°C with colloidal gold particles (10 to 15 nm in diameter) conjugated with goat antibodies to rabbit immunoglobulin G. After five 30-min washes in PBS, the agarose-embedded membranes were fixed for 30 min with 1% glutaraldehyde in PBS, washed in 0.1 M sodium cacodylate buffer (pH 7.4) (two times, 5 min each), incubated for 1 hour with 1% OsO_4, dehydrated, and embedded in Epon. Ultrathin sections were cut, stained with 1% lead citrate, and viewed under a Philips P-300 electron microscope.
46. The silica-coated membrane pellet already stripped of caveolae (P–V) was resuspended in 20 mM 2-(*N*-morpholino)ethanesulfonic acid with 125 mM NaCl and an equal volume of 4 M K_2HPO_4 and 0.2% polyacrylate (pH 9.5). The solution was sonicated (10 10-s bursts) with cooling, mixed on a rotator for 8 hours at room temperature (20° to 25°C), and sonicated again (five 10-s bursts). Triton X-100 was added to 1%, and the preparation was then mixed for 10 min at 4°C and homogenized with a Type AA Teflon tissue grinder (Thomas Scientific, Swedesboro, NJ). Any intact floating detergent-resistant membranes were separated and isolated from this homogenate by sucrose density gradient centrifugation as in (*13*). The pellet, containing silica and any remaining membranes, was also collected.
47. Cholera toxin B fragment (CT) at 60 μg/ml was conjugated to 15-nm colloidal gold in 2 mM sodium borate buffer (pH 6.9) for 30 min with stirring. Polyethylene glycol (20 kD) was added for the last 5 min to a final concentration of 0.5 mg/ml. Unconjugated CT was removed by three cycles of centrifugation (10,000*g* for 20 min) and resuspension in 5 mM sodium phosphate buffer (pH 7.2). The resuspended conjugate (CT-Au) was dialyzed overnight against a solution containing 50 mM tris (pH 6.9) and 150 mM NaCl. Sodium azide was added to 0.02% and the conjugate was stored at 4°C. The isolated membranes were labeled as described (*45*), with the exception that CT-Au replaced the primary antibody and was followed directly by washing without additional incubations.
48. We thank J. P. Luzio for providing the antibodies to 5′-NT and W. S. Sly for the polyclonal antibodies to CA. Supported by NIH grants HL43278 and HL52766 (J.E.S.) and AI33372 (A.M.D.), and an Established Investigator Award from the American Heart Association and Genentech (J.E.S.).

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Elongin (SIII): A Multisubunit Regulator of Elongation by RNA Polymerase II

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The Elongin (SIII) complex activates elongation by mammalian RNA polymerase II by suppressing transient pausing of the polymerase at many sites within transcription units. Elongin is a heterotrimer composed of A, B, and C subunits of 110, 18, and 15 kilodaltons, respectively. Here, the mammalian Elongin A gene was isolated and expressed, and the Elongin (SIII) complex reconstituted with recombinant subunits. Elongin A is shown to function as the transcriptionally active component of Elongin (SIII) and Elongin B and C as regulatory subunits. Whereas Elongin C assembles with Elongin A to form an AC complex with increased specific activity, Elongin B, a member of the ubiquitin-homology gene family, appears to serve a chaperone-like function, facilitating assembly and enhancing stability of the Elongin (SIII) complex.

Eukaryotic messenger RNA synthesis is a complex biochemical process controlled in part by the concerted action of a set of general transcription factors that regulate the activity of RNA polymerase II (Pol II) at both the initiation and elongation stages of transcription. At least six general initiation factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) 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, three general elongation factors [SII, TFIIF, and Elongin (SIII)] from eukaryotes have been defined biochemically and shown to increase the overall rate at which Pol II transcribes duplex DNA (*2–4*). SII is an ~38-kD elongation factor (*5*) that promotes passage of Pol II through transcriptional impediments such as nucleoprotein complexes and DNA sequences that act as intrinsic arrest sites. SII-dependent readthrough is accompanied by reiterative transcript cleavage and reextension of nascent transcripts held in the Pol II active site (*6–9*).

TFIIF from higher eukaryotes is a heterodimer composed of ~70-kD (RAP74)

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and ~ 30-kD (RAP30) subunits (*1*). In the yeast *Saccharomyces cerevisiae*, TFIIF is a heterotrimer, two subunits of which are homologs of RAP74 and RAP30 (*10, 11*). Elongin (SIII) is a heterotrimer composed of an A subunit (~110 kD), a B subunit (~18 kD), and a C subunit (~15 kD) (*12, 13*). In contrast to SII, Elongin (SIII) and TFIIF neither release Pol II from transcriptional arrest at SII-sensitive sites in duplex DNA nor induce nascent transcript cleavage. Instead, they appear to function by suppressing transient pausing of Pol II at many sites within transcription units (*13, 14*).

In previous studies, we isolated full-length complementary DNAs (cDNAs) encoding the Elongin B and C subunits (*15–17*). Here, we isolated the cDNA for the Elongin A subunit and describe the functional reconstitution of Elongin (SIII) from the three recombinant subunits.

Elongin (SIII) was purified to near homogeneity from rat liver nuclear extracts (*12*). Elongin A was purified free of Elongin B and C by reversed-phase high-performance liquid chromatography (HPLC) and digested with trypsin. The NH_2-terminal sequences of six tryptic peptides were determined by sequential Edman degradation (*18*). A rat brain cDNA library was screened with a degenerate oligonucleotide (guessmer) designed from a region of low codon degeneracy (*19*). Phage clones containing Elongin A cDNA sequences were identified by the polymerase chain reaction (PCR) with one primer complementary to the vector sequence and a second guessmer primer derived from the sequence of an Elongin A tryptic peptide. The Elongin A cDNA contains an open reading frame (ORF) encoding a protein of 773 amino acids with a calculated molecular mass of 87.2 kD; the predicted amino acid sequence includes the sequences of all six tryptic peptides (Fig. 1A).

The Elongin A ORF was subcloned into an M13 expression vector (*20*) under the control of the T7 RNA polymerase promoter and expressed in *Escherichia coli* (*21*). Recombinant Elongin A had an apparent molecular mass of 110 kD and an electrophoretic mobility indistinguishable from that of rat liver Elongin A (Fig. 1B, lanes 1 and 2). Polyclonal antisera raised against a synthetic peptide corresponding to Elongin A amino acids 100 to 115 recognized (*22*) both recombinant Elongin A and rat liver Elongin A (Fig. 1B, lanes 3 to 6).

Elongin (SIII) increases the overall rate of RNA chain elongation by Pol II in a reaction that is strongly dependent on the Elongin A subunit (*12*). To test the activity of recombinant Elongin A, we assembled Elongin (SIII) complexes by renaturation of different combinations of individual Elongin subunits (*12*) and assayed their ability to increase the rate of accumulation of full-length runoff transcripts synthesized from the adenovirus 2 major late (AdML) promoter (*17*). In these experiments, preinitiation complexes were assembled at the AdML promoter, the renatured Elongin subunits or the native Elongin (SIII) complex from rat liver was added to the reaction mixtures, and transcription was initiated by addition of limiting concentrations of ribonucleoside triphosphates (Fig. 1C). Under these conditions, the rate of RNA chain elongation is very slow and full-length runoff transcripts do not accumulate unless elongation stimulatory activity is present. Approximately equal amounts of runoff transcripts were synthesized in reactions containing native Elongin (SIII) complex and those containing the fully reconstituted Elongin (SIII) complex. In contrast, there was no detectable stimulation of runoff transcription in reactions containing Elongin B and C that had been renatured in the absence of Elongin A.

Although Elongin A contains no structural motifs characteristic of transcription factors, such as zinc finger, leucine zipper, or helix-turn-helix domains, its NH_2-termi-

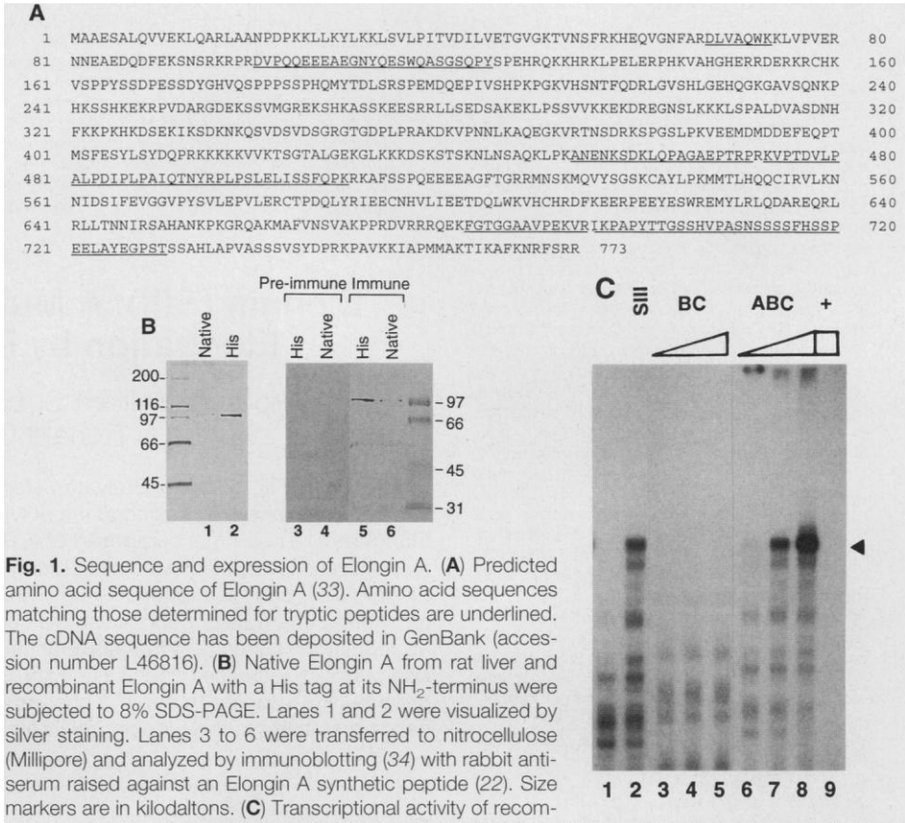


Fig. 1. Sequence and expression of Elongin A. (**A**) Predicted amino acid sequence of Elongin A (*33*). Amino acid sequences matching those determined for tryptic peptides are underlined. The cDNA sequence has been deposited in GenBank (accession number L46816). (**B**) Native Elongin A from rat liver and recombinant Elongin A with a His tag at its NH_2-terminus were subjected to 8% SDS-PAGE. Lanes 1 and 2 were visualized by silver staining. Lanes 3 to 6 were transferred to nitrocellulose (Millipore) and analyzed by immunoblotting (*34*) with rabbit antiserum raised against an Elongin A synthetic peptide (*22*). Size markers are in kilodaltons. (**C**) Transcriptional activity of recombinant Elongin A. Various combinations of recombinant Elongin (SIII) subunits were renatured together and assayed for transcriptional activity (*17*). Briefly, ~150 ng of His–Elongin A (*21*), ~30 ng of His–Elongin B (*17*), and ~30 ng of His–Elongin C (*15*), all in 6 M guanidine-HCl denaturing buffer, were mixed together in a total of 5 μl and diluted with 45 μl of renaturing buffer. After 90 min on ice, the renatured proteins were dialyzed for ~2 hours against 40 mM Hepes-NaOH (pH 7.9), 0.1 M KCl, 50 μM $ZnSO_4$, and 10% (v/v) glycerol. Reaction mixtures contained ~20 ng of rat Elongin (SIII) (lane 2); 1, 3, and 9 μl of renatured Elongin B and C (lanes 3 to 5); and 1, 3, 9, and 9 μl of renatured Elongon A, B, and C (lanes 6 to 9). The reaction shown in lane 9 contained 1 μg of α-amanitin per milliliter. Arrowhead indicates position of the full-length (250-nucleotide) runoff transcript synthesized from the AdML promoter. Lane 1, no Elongin.

Fig. 2. Sequence similarity between Elongin A (El A) and human SII (*33, 35*), identified with the GAP program of the Genetics Computer Group package (*36*); lines, double dots, and dots are as described (*36*).

```
SII   2 EDEVVRFAKKMDKMVQKKNAAGALDLLKELKNIPMTLELLQSTRIGMSVN 51
          |..:  ..|  .:::....::   |..||.|. :|:|:::| .| :|..||
El A  4 ESALQVVEKLQARLAANPDPKKLLKYLKKLSVLPITVDILVETGVGKTVN 53
                .        .        .        .        .
     52 AIRKQSTDEEVTSLAKSLIKSWKKLL....DGPSTEKDLD..EKKKEPAI 95
          .:||:   |:|..:|:.|:  ||||:    :.....:|::  :.:| |
     54 SFRKH...EQVGNFARDLVAQWKKLVPVERNNEAEDQDFEKSNSRKRPRD 100
                .        .
     96 TSQNSPEAREESTSSGNVSN 115
          ..|...|| ::  .| ..|.
    101 VPQQEEEAEGNYQESWQASG 120
```

nus shares sequences with the NH_2-terminus of elongation factor SII (Fig. 2). The two proteins are 29% identical over a 108–amino acid overlap; when conservative amino acid substitutions are included, the sequence similarity is 53%, which suggests that SII and Elongin A are members of a family of related transcription factors. Interestingly, the region of similarity is dispensable for the activities of both SII and Elongin A in vitro (*9*, *23*); however, the conservation of this large NH_2-terminal region suggests that it plays an in vivo regulatory role not yet revealed by in vitro assays.

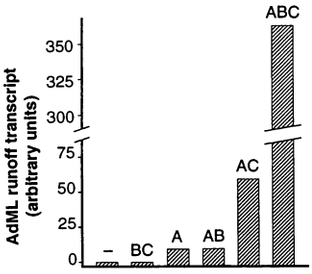


Fig. 3. Reconstitution of transcriptionally active Elongin (SIII) and Elongin subassemblies. Various combinations of His–Elongin A (~150 ng), His–Elongin B (~30 ng), and His–Elongin C (~30 ng) were renatured together, and 9 μl of each renaturation mix was assayed for transcriptional activity. Synthesis of full-length AdML runoff transcripts was quantitated by densitometry of autoradiograms.

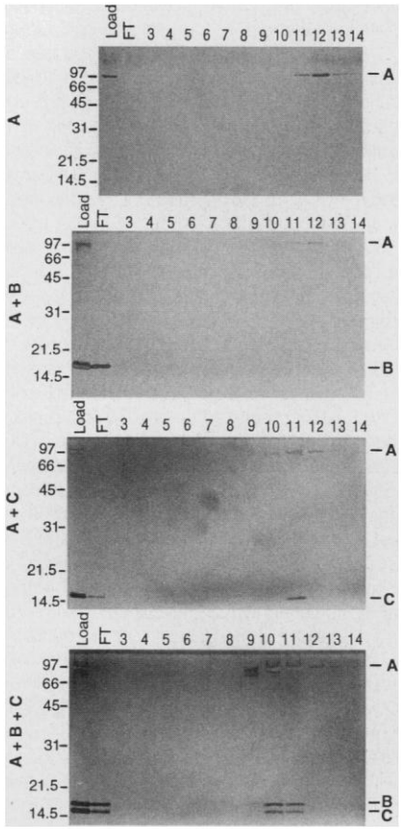


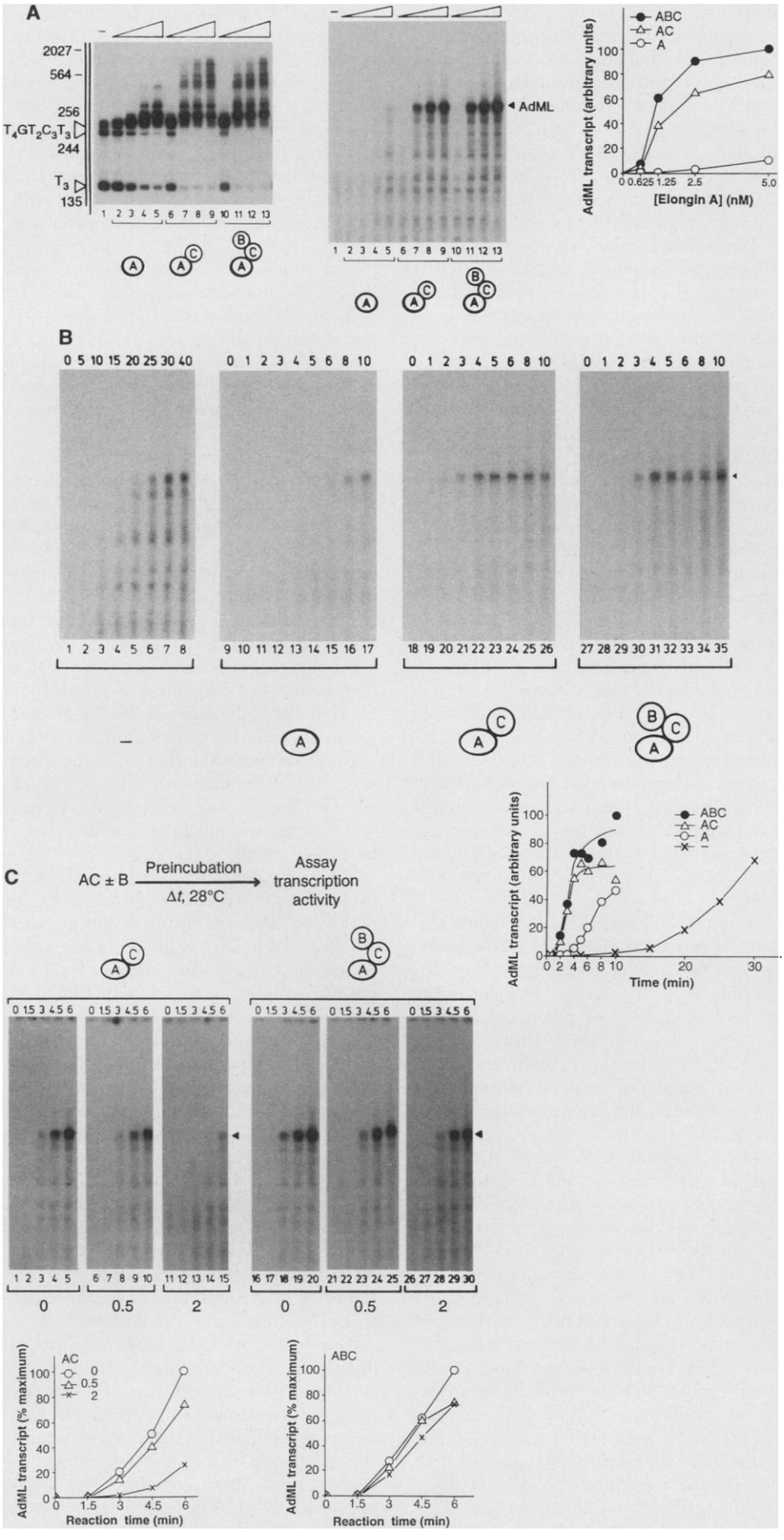
Fig. 4. Isolation of Elongin (SIII) and Elongin subassemblies. The indicated combinations of Elongin subunits were renatured together and analyzed by HPLC on TSK SP-NPR (*24*). The column fractions were analyzed by SDS-PAGE, and the proteins detected by silver staining. Size markers are shown on the left in kilodaltons. FT, flow through.

To assess the individual contributions of Elongin A, B, and C to Elongin (SIII) transcriptional activity, we renatured the subunits together in various combinations and measured the activity of the resultant complexes in the AdML runoff transcription assay (*17*) (Fig. 3). Because we tested equivalent volumes of each renaturation mix, these assays measured the recovery, but not specific activity, of Elongin after renaturation. Maximal Elongin activity was recovered only when the A, B, and C subunits were renatured together. Elongin A exhibited a small amount of transcription activity when it was renatured in the absence of the B and C subunits, but the B and C subunits had no detectable activity in the absence of Elongin A (Figs. 1C and 3). Addition of the B subunit to Elongin A had no detectable effect on the recovery of Elongin activity, whereas addition of the C subunit to Elongin A resulted in an increased recovery of activity; thus, Elongin B activity is dependent on the presence of Elongin C. Together, these results suggest that Elongin A is the transcriptionally active component of the Elongin (SIII) complex, whereas Elongin B and C are positive regulatory subunits.

In principle, Elongin B and C could regulate the activity of Elongin by (i) increasing the specific activity of Elongin A or (ii) functioning as "chaperones" to increase the recovery or stability of functional Elongin complexes. To distinguish between these possibilities, we renatured Elongin A alone or in the presence of various combinations of Elongin B and C and determined the specific activity of the resultant complexes in transcription assays (*24*). To purify the intact Elongin (SIII) complex and Elongin subassemblies, we took advantage of the fact that Elongin B and C are acidic proteins that flow through the cation-exchange resin TSK SP-NPR at low salt concentrations, whereas Elongin A, like the native Elongin (SIII) from rat liver (*12*), binds tightly to this resin and can be eluted at high concentrations of salt (FIg. 4, top). Upon renaturation and cation-exchange chromatography of a mixture of the Elongin A, B, and C subunits, excess B and C subunits flowed through the column, and the remainder copurified with Elongin A to reconstitute an ABC complex resembling the native Elongin (SIII) complex (Fig. 4, A + B + C). Upon renaturation and chromatography of a mixture of the Elongin A and C subunits, a substantial portion of the C subunit copurified with Elongin A as an AC complex that eluted from the column in a single, discrete peak (Fig. 4, A + C). The total yield of Elongin A recovered under these conditions, however, was significantly less than when renaturation mixes also contained Elongin B. The ABC complexes contained about three times more Elongin A than the AC complexes, even though equal amounts of Elongin A were used in the initial renaturations. Consistent with our observation that the B subunit does not affect Elongin A activity unless the C subunit is also present (Fig. 3), the B subunit did not copurify with Elongin A upon renaturation and chromatography of a mixture of Elongin A and B (Fig. 4, A + B).

The specific activities of purified Elongin A and the Elongin AC and ABC complexes were compared in the AdML runoff transcription assay (*17*) and the oligo(dC)-tailed template assay (*25*, *26*). The latter permits a direct measurement of the effect of Elongin on the rate of RNA chain elongation by Pol II in the absence of other transcription factors. The specific activities of the AC and ABC complexes were nearly identical in both assays (Fig. 5A). Thus, although Elongin B increases the recovery of Elongin complexes and Elongin transcription activity, it makes little or no contribution to the specific activity of the Elongin (SIII) complex. In contrast, the specific activity of the Elongin AC complex was much greater than that of Elongin A alone.

These findings were confirmed by an experiment in which we measured the kinetics of AdML runoff transcription in the absence of Elongin or in the presence of equimolar amounts of Elongin A, the Elongin AC complex, or the Elongin ABC complex (Fig. 5B). In the absence of Elongin, full-length runoff transcripts were first detected ~15 min after addition of ribonucleoside triphosphates (Fig. 5B, lanes 1 to 8). With addition of Elongin A, transcripts accumulated at an intermediate rate, with the first full-length runoff transcripts appearing within 4 or 5 min (lanes 9 to 17). With the addition of either the Elongin AC or ABC complex, transcripts accumulated more rapidly, with the first full-length runoff transcripts appearing within ~2 min (lanes 18 to 26 and lanes 27 to 35, respectively). At early time points, the rate of accumulation of runoff transcripts was indistinguishable in reactions containing the AC or ABC complex (Fig. 5B). At later time points, however, more runoff transcripts accumulated in reactions containing the complete ABC complex than in those containing the AC complex. This difference was small, but highly reproducible, and suggested that the ABC complex was



more stable than the AC complex over the time course of transcription reactions.

To test this possibility, we investigated the effect of the B subunit on the thermal stability of Elongin. Purified AC and ABC complexes were preincubated at 28°C for either 30 min or 2 hours and their activity was then measured in the AdML runoff transcription assay. The complete Elongin ABC complex lost little or no activity during 2 hours of preincubation, whereas the AC complex lost substantial activity during this period (Fig. 5C). Taken together, these results indicate that Elongin B both facilitates the assembly and enhances the stability of the Elongin (SIII) complex.

Thus, Elongin B and C appear to regulate the activity of the Elongin A subunit by different mechanisms. Elongin C, a 112–amino acid protein with homology to the *E. coli* transcription termination protein rho (*15*), binds stably to Elongin A in the absence of Elongin B to form an AC complex with increased specific activity. This suggests

Fig. 5. Characterization of the regulatory activities of Elongin B and C. (**A**) Comparison of specific activities of Elongin A, Elongin AC, and Elongin ABC complexes in oligo(dC)-tailed template assays (left) and runoff transcription assays (middle and right). Reaction mixtures in lanes 2 to 5 contained 0.6, 1.2, 2.5, and 5 nM purified Elongin A (fraction 12, top panel of Fig. 4). Reactions in lanes 6 to 9 contained 0.6, 1.2, 2.5, and 5 nM purified Elongin AC complex (fraction 11, third panel of Fig. 4). Reactions in lanes 10 to 13 contained 0.6, 1.2, 2.5, and 5 nM purified Elongin ABC complex (fraction 11, bottom panel of Fig. 4). The full-length runoff transcripts were quantitated (right) by phosphorimager analysis of the gel. Numbers on the left indicate DNA lengths. (**B**) Comparison of the kinetics of AdML (arrowhead) runoff transcript synthesis in the presence of Elongin A and Elongin AC and ABC complexes. After a 30-min preincubation of Pol II, transcription factors, and DNA template, transcription was carried out for the indicated times (top, in minutes) in the absence (lanes 1 to 8) or presence of 5 nM Elongin A (lanes 9 to 17), 5 nM Elongin AC (lanes 18 to 26), or 5 nM Elongin ABC (lanes 27 to 35). Full-length runoff transcripts were quantitated by phosphorimager analysis (lower right). (**C**) Effect of Elongin B on the thermal stability of the Elongin (SIII) complex. Arrowheads indicate AdML. Elongin AC or ABC complexes were preincubated at 28°C for 30 min or 2 hours, and their activities were compared in the runoff transcription assay to the activity of untreated complexes. Reaction times are at the top, in minutes; preincubation times are at the bottom, in hours. The assay was carried out as in (B) in the presence of 5 nM untreated AC complex (lanes 1 to 5), 5 nM untreated ABC complex (lanes 16 to 20), 5 nM AC complex preheated for 30 min (lanes 6 to 10), 5 nM ABC complex preheated for 30 min (lanes 21 to 25), 5 nM AC complex preheated for 2 hours (lanes 11 to 15), or 5 nM ABC complex preheated for 2 hours (lanes 26 to 30). Full-length runoff transcripts were quantitated by phosphorimager analysis (bottom panels).

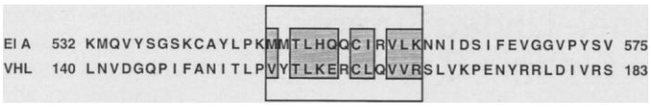


Fig. 6. Sequence similarity between Elongin A (EI A) and VHL (*33*). Similar or identical amino acids are indicated by shaded boxes.

that Elongin C functions as a direct activator of Elongin A. Whether it increases Elongin A activity solely through conformational effects or is directly involved in interactions with Pol II, the nascent transcript, template DNA, or some combination of these components of the ternary elongation complex is unknown. Elongin B, a 118–amino acid protein, is a member of the ubiquitin homology (UbH) gene family (*17*). Unlike Elongin C, Elongin B does not increase the specific activity of Elongin A but instead appears to play a chaperone-like role in promoting the assembly and thermostability of the Elongin (SIII) complex. A chaperone-like role for ubiquitin is not unprecedented: Ubiquitin moieties of several *S. cerevisiae* ribosomal proteins function as chaperones that promote the incorporation of these proteins into ribosomes (*27*). In addition, *S. cerevisiae* RAD23, a UbH protein involved in nucleotide excision repair, promotes interactions between RAD14 and TFIIH (*28*).

Finally, the discovery that the von Hippel–Lindau (VHL) tumor suppressor protein binds to Elongin B and C and inhibits Elongin (SIII) activity in vitro (*29*) raises the possibility that Elongin (SIII) may be an integral component of a transcriptional regulatory network controlled at least in part by the VHL protein. It is noteworthy that residues 547 to 560 of Elongin A are similar to a region of VHL that is frequently mutated in VHL kindreds and in sporadic renal cell carcinomas (*30*) (Fig. 6). This is the only region in VHL with significant sequence similarity to Elongin A and is the region required for binding to Elongin B and C (*31*). This finding, together with the observation that excess VHL can prevent Elongin B and C subunits from assembling into the Elongin ABC complex (*29*), suggest that VHL may inhibit Elongin (SIII) activity simply by sequestering Elongin B and C. Elucidation of the mechanism by which VHL regulates Elongin activity should provide insight into the molecular basis of VHL tumor suppressor action.

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18. Approximately 300 pmol of Elongin A was isolated by reversed-phase HPLC (*12*). After reduction, *S*-carboxyamidomethylation, and digestion with trypsin, the resultant mixture was further fractionated by microbore HPLC. Optimal peptides were determined by differential absorbance of ultraviolet light and matrix-assisted laser desorption mass spectrometry (Lasermat; Finnigan-MAT, San Jose, CA). The peptides were then sequenced by automated Edman degradation (*32*).
19. Two of the amino acid sequences, DVPQQEEEAEGNYQESWQASGSQPY and ANENKSDKLQPAGAEPTRP (*33*), were used to synthesize oligonucleotide guessmers (primer I, 5′-TCCTGGTAGTTICCCTCIGCCTCCTCCTCCTGCTGIGGIACGTC-3′, and primer II, 5′-CGGATCCGTIGG(T/C)TCIGCICCIGCIGG(T/C)TG-3′, respectively), according to the rat codon usage bias. Inosine was used instead of a four-nucleotide mixture. A λ-GEM2 library constructed from rat brain cDNA was screened with 5′ ^{32}P-labeled primer I. Hybridization was performed at 37°C for 20 hours in 6× standard saline citrate, 1× Denhardt's solution, and 0.05% sodium pyrophosphate containing denatured salmon testis DNA (100 μg/ml). A total of 69 phage clones were isolated from 5×10^5 plaques. Positive clones from oligonucleotide screening were identified by PCR. The sense and antisense primers were T7 primer and primer II, respectively. PCR was performed for 30 cycles for 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. Of 69 phage clones, eight were positive by PCR screening. Two independently isolated cDNA clones of 3.6 kilobases (kb) and 3.5 kb were subcloned into pUC18, and the inserts were sequenced on both strands. The two clones were identical in sequence except that one was 105 base pairs longer at the 5′ end.
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21. Elongin A was overexpressed in *E. coli* with an M13mpET bacteriophage vector (*20*). Constructs for expression of histidine (His)-tagged Elongin A were prepared by insertion of PCR-generated fragments of Elongin A cDNA into the Sal I and Bam HI sites of M13mpET, which contains the complete pET T7 transcription-translation region as well as sequences encoding the His tag. PCR fragments were generated with primers encoding the amino acids at both ends of each fragment. The primers also contained extra nucleotides comprising a Sal I site at one 5′ end and a Bam HI site at the other 5′ end. For preparation of recombinant Elongin A, a 100-ml culture of *E. coli* strain JM109(DE3) was grown to an absorbance (at 600 nm) of 0.6 with gentle shaking in Luria broth medium containing 2.5 mM $MgCl_2$ at 37°C. Cells were infected with M13mpET carrying the Elongin A cDNA at a multiplicity of infection of 20. After 2 hours at 37°C, cells were induced with 0.4 mM isopropyl β-D-thiogalactoside and the culture was incubated for an additional 2.5 hours. Cells were harvested by centrifugation at 2000*g* for 10 min at 4°C. Inclusion bodies (*15*, *17*) were solubilized by resuspension in 5 ml of ice-cold 50 mM tris-HCl (pH 8.0) containing 6 M guanidine-HCl. The suspension was clarified by centrifugation at 50,000*g* for 20 min at 4°C. His–Elongin A was purified from the supernatant by nickel chromatography on ProBond resin (*15*). After renaturation and dialysis, the Elongin A preparation was centrifuged at 60,000*g* for 20 min at 4°C, and the supernatant was applied to a Bio-Gel TSK SP-5PW column (7.5 mm by 75 mm) pre-equilibrated with 40 mM Hepes-NaOH (pH 7.9), 0.1 M KCl, 1 mM dithiothreitol (DTT), 50 μM $ZnSO_4$, 0.1 mM EDTA, and 10% (v/v) glycerol. The column was eluted at 1 ml/min with a 50-ml linear gradient from 0.1 to 0.8 M KCl in the same buffer. Fractions (1 ml) were collected. Elongin A eluted between 0.35 and 0.40 M KCl.
22. The synthetic peptide (amino acids 100 to 115 of rat Elongin A plus an NH_2-terminal CGG) was coupled to maleimide-activated ovalbumin with use of an immunoject activated immunogen conjugation kit (Pierce) according to the manufacturer's instructions. The resultant conjugate (0.2 mg) was injected in complete Freund's adjuvant into a rabbit. The rabbit was boosted at 4-week intervals with 0.2 mg of conjugated peptide in complete Freund's adjuvant.
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24. Renaturation of the recombinant Elongin subunits was carried out essentially as in (*12*, *17*) with 100 μg of His–Elongin A, 15 μg of His-Elongin B (*17*), and 15 μg of His–Elongin C (*15*). After dialysis, the renatured proteins were applied to a TSK SP-NPR column (35 mm by 4.6 mm, Hewlett-Packard) equilibrated in 40 mM Hepes-NaOH (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 0.1 M KCl. The column was eluted at 0.6 ml/min at 8°C with a 9-ml gradient from 0.1 to 0.8 M KCl in the same buffer. Samples of each column fraction were analyzed by 12% SDS–polyacrylamide gel electrophoresis (PAGE) and the proteins visualized by silver staining.
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26. Pol II (0.01 unit) and pCpGR220A/P/X (100 ng) were incubated at 28°C in the presence of 200 mM Hepes, 20 mM tris-HCl (pH 7.9), 2% (w/v) polyvinyl alcohol, bovine serum albumin (0.5 mg/ml), 60 mM KCl, 50 μM $ZnSo_4$, 7 mM $MgCl_2$, 0.2 mM DTT, 3% (v/v) glycerol, 3 units of recombinant RNasin (Promega), 50 μM adenosine triphosphate, 50 μM guanosine triphosphate, 2 μM cytosine triphosphate (CTP), and 10 μCi of [α-^{32}P]CTP. After 25 min, 10 μM nonradioactive CTP, 2 μM uridine triphosphate, and the Elongin preparations were added, and the reactions incubated for 7.5 min further. Transcripts were analyzed by electrophoresis through 6% polyacrylamide, 7.0 M urea gels.
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33. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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37. We thank K. Garrett for the Elongin B and C clones and for support; D. Haque and Y. Takagi for help with purification of recombinant proteins; R. Robinson for peptide isolation; K. Payne for DNA sequencing; J. Minnerley and R. Wiegand for the rat brain cDNA library; P. Silverman, R. Klausner, A. Pause, R. Duan, G. Zurawski, S. Zurawski, and D. Liggett for helpful discussions; and K. Jackson for oligonucleotide synthesis. Supported by NIH grant GM41628 and by funds provided to the Oklahoma Medical Research Foundation by the H. A. and Mary K. Chapman Charitable Trust.

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