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# The inducible elongin A elongation activation domain: structure, function and interaction with the elongin BC complex

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**The elongin (SIII) complex strongly stimulates the rate of elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites along the DNA. Elongin (SIII) is composed of a transcriptionally active A subunit and two small regulatory B and C subunits, which bind stably to each other to form a binary complex that interacts with elongin A and strongly induces its transcriptional activity. The elongin (SIII) complex is a potential target for negative regulation by the von Hippel–Lindau (VHL) tumor suppressor protein, which is capable of binding stably to the elongin BC complex and preventing it from activating elongin A. Here, we identify an elongin A domain sufficient for activation of elongation and demonstrate that it is a novel type of inducible activator that targets the RNA polymerase II elongation complex and is evolutionarily conserved in species as distantly related as *Caenorhabditis elegans* and man. In addition, we demonstrate that both the elongin A elongation activation domain and the VHL tumor suppressor protein interact with the elongin BC complex through a conserved elongin BC binding site motif that is essential for induction of elongin A activity by elongin BC and for tumor suppression by the VHL protein.**

*Keywords*: elongation activation domain/elongin/RNA polymerase II/von Hippel–Lindau tumor suppressor protein

## Introduction

Eukaryotic mRNA synthesis by RNA polymerase II is 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 (Conaway and Conaway, 1993; Krumm *et al.*, 1993; Kane, 1994; Aso *et al.*, 1995a). At least five general initiation factors (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 (Conaway and Conaway, 1993). In addition, five general elongation factors [SII, P-TEFb, TFIIF, ELL and elongin (SIII)] have been defined biochemically and found to increase the efficiency of elongation by RNA polymerase II.

Of the general elongation factors, SII and P-TEFb prevent RNA polymerase II from arresting transcription prematurely. SII prevents arrest 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 (Reines, 1994). P-TEFb catalyzes the conversion of early, termination-prone elongation complexes into productive elongation complexes (Marshall and Price, 1992, 1995). The remaining elongation factors, TFIIF (Price *et al.*, 1989), ELL (Shilatifard *et al.*, 1996) and elongin (SIII) (Bradsher *et al.*, 1993a,b) all act to increase the overall rate of elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites within transcription units. A variety of evidence implicates two of these elongation factors in the development of certain types of human cancers. The gene encoding ELL is a frequent target for t(11;19) chromosomal translocations in acute myeloid leukemias (Thirman *et al.*, 1994; Mitani *et al.*, 1995). 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 (Duan *et al.*, 1995b; Kibel *et al.*, 1995).

Elongin (SIII) was initially purified from mammalian cells as a multimeric complex composed of A, B and C subunits of 773, 118 and 112 amino acids, respectively (Bradsher *et al.*, 1993a; Garrett *et al.*, 1994, 1995; Aso *et al.*, 1995b). Elongin A is the transcriptionally active subunit of the elongin (SIII) complex, whereas elongin B and C are regulatory subunits. Biochemical studies have shown that elongin B and C bind stably to each other in the absence of elongin A to form a binary complex that is capable of strongly inducing elongin A transcriptional activity (Aso *et al.*, 1995b; Garrett *et al.*, 1995). By virtue of its ability to bind directly to elongin A in the absence of elongin B to form an elongin AC complex with increased specific activity, elongin C appears to be responsible for induction of elongin A activity. In contrast, 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 of the elongin (SIII) complex by binding directly to elongin C and promoting its interaction with elongin A.

Evidence that elongin (SIII) is a potential target for regulation by the VHL tumor suppressor protein has come from recent results indicating that the VHL protein binds tightly and specifically to the elongin BC complex *in vivo* and *in vitro* (Duan *et al.*, 1995a,b; Kibel *et al.*, 1995).

The interaction of the VHL protein with the elongin BC complex is mediated at least in part by a short VHL region that is similar in sequence to a region of elongin A (Kibel *et al.*, 1995). Supporting the idea that the VHL–elongin BC interaction is important for VHL protein tumor suppressor activity, a significant fraction of all naturally occurring VHL mutants found in VHL kindreds and in clear-cell renal carcinoma have mutations that fall within this conserved region, and a number of these VHL mutants have been tested and found to exhibit reduced binding to elongin BC. Binding of the VHL protein and elongin A to the elongin BC complex is mutually exclusive *in vitro*. Furthermore, binding of the VHL protein to the elongin BC complex blocks its ability to activate elongin A transcriptional activity. Thus, the normal tumor suppressor function of the VHL protein could involve down-regulation of elongin (SIII) transcriptional activity.

As part of our effort to understand how the elongin BC complex regulates the activity of elongin A, we have identified and carried out a systematic structure–function analysis of the elongin A elongation activation domain. The results of these studies define a new class of inducible activation domain evolutionarily conserved in species as distantly related as *Caenorhabditis elegans* and man. In addition, they define a new protein–protein interaction motif by demonstrating that both the elongin A elongation activation domain and the VHL tumor suppressor protein interact with the elongin BC complex through a highly conserved elongin BC binding site that is essential for induction of elongin A activity by elongin BC and for tumor suppression by the VHL protein.

## Results

### *Localization of the elongin A elongation activation domain and identification of the elongin BC binding site*

Elongin A is a 773 amino acid protein with a calculated $M_r$ of 87.2 kDa (Aso *et al.*, 1995b). Our previous analysis of the predicted open reading frame (ORF) of the elongin A cDNA revealed several notable features that suggested the existence of potentially important elongin A functional domains. First, the N-terminal ~110 amino acids of elongin A resemble the N-terminus of elongation factor SII (29% identity and 53% similarity to human SII) (Chen *et al.*, 1992). Second, as revealed by a MOTIFs search (Genetics Computer Group, 1994), both rat and human elongin A have potential ATP binding sites (residues 44–51) (Walker *et al.*, 1982; Gorbalenya *et al.*, 1989), as well as the sequence NSKMQVYSGSKCAYLPKMM (residues 530–549), which differs by only a single residue from the consensus topoisomerase I catalytic site motif (D,E)x6-(G,S)xSKx2Y(L,I,V,M)x3(L,I,V,M) (Eng *et al.*, 1989; Lynn *et al.*, 1989). Third, both the Chou–Fasman (Chou and Fasman, 1974) and Garnier (Garnier *et al.*, 1978) algorithms predict that the C-terminus of elongin A between residues 749 and 766 will form an α-helix that is rich in hydrophobic amino acids on one face of the helix and, therefore, has the potential to form a coiled coil protein–protein interaction domain analogous to those found in leucine zipper sequences (Landschulz *et al.*, 1988; O'Shea *et al.*, 1989). Finally, elongin A contains a short sequence (residues 547–560) that resembles the elongin BC binding site in the VHL tumor suppressor protein (Kibel *et al.*, 1995).

To localize the elongin A elongation activation domain and to identify the elongin BC binding site, a series of N- and C-terminal elongin A deletion mutants (Figure 1A) were constructed, expressed in *Escherichia coli*, purified and assayed for their abilities (i) to assemble into chromatographically isolable elongin ABC complexes and (ii) to stimulate the rate of elongation of transcripts synthesized by RNA polymerase II from the adenovirus major late (AdML) promoter in a reconstituted basal transcription system composed of TBP and the general initiation factors TFIIB, TFIIE, TFIIF and TFIIH. To test the abilities of elongin A mutants to bind elongin B and C and to isolate mutant elongin ABC complexes in order to assay their relative transcriptional activities, N- and C-terminal elongin A deletion mutants were refolded together with wild-type elongin B and C and subjected to cation exchange HPLC on a TSK SP–NPR column (Toso-Haas). Wild-type and mutant elongin ABC complexes bind tightly to TSK SP-NPR and elute at salt concentrations between 0.2 and 0.4 M KCl, whereas excess elongin B and C flow through this resin at 0.1 M KCl (Aso *et al.*, 1995b). Figure 1B shows SDS–PAGE analysis of aliquots of peak column fractions from TSK SP-NPR HPLC purification of mutant elongin ABC complexes. Results of this analysis revealed that all N- and C-terminal elongin A deletion mutants except A(1–535), which lacks the region similar to the elongin BC binding site in the VHL protein (Kibel *et al.*, 1995), were able to bind stably to elongin B and C to form isolable elongin ABC complexes.

The relative transcriptional activities of the wild-type and mutant elongin ABC complexes shown in Figure 1B were then compared in run-off transcription assays. Pre-initiation complexes were assembled at the AdML promoter, wild-type and mutant elongin ABC complexes were added to reaction mixtures, and transcription was initiated by addition of limiting concentrations of ribonucleoside triphosphates. Under these conditions, the rate of RNA chain elongation is very slow, and run-off transcripts do not accumulate unless elongation stimulatory activity is present (Bradsher *et al.*, 1993b; Aso *et al.*, 1995b; Figure 1C, lanes 1 and 2). Results of these experiments indicated that the first 399 elongin A amino acids, which include the region of SII similarity and the potential ATP binding site, are dispensable for elongin ABC activity *in vitro*, since deletion of this region had no significant effect on accumulation of run-off transcripts. In contrast, deletion of the C-terminal 93 amino acids, which include the potential hydrophobic zipper, decreased elongin ABC activity by ~50%, and deletion of the C-terminal 143 amino acids abolished detectable elongin ABC activity. Taken together, these results identified: (i) a 231 amino acid elongin A region (residues 400–630), which includes sequences required for interaction with elongin B and C; (ii) a 281 amino acid elongin A region (residues 400–680), which includes sequences required for transcriptional activity; and (iii) an additional C-terminal elongin A region (residues 680–773), which includes sequences important for maximal transcriptional activity.

To define further the elongin A sequences required for binding to elongin B and C and for transcriptional activity, a series of elongin A internal deletion mutants with

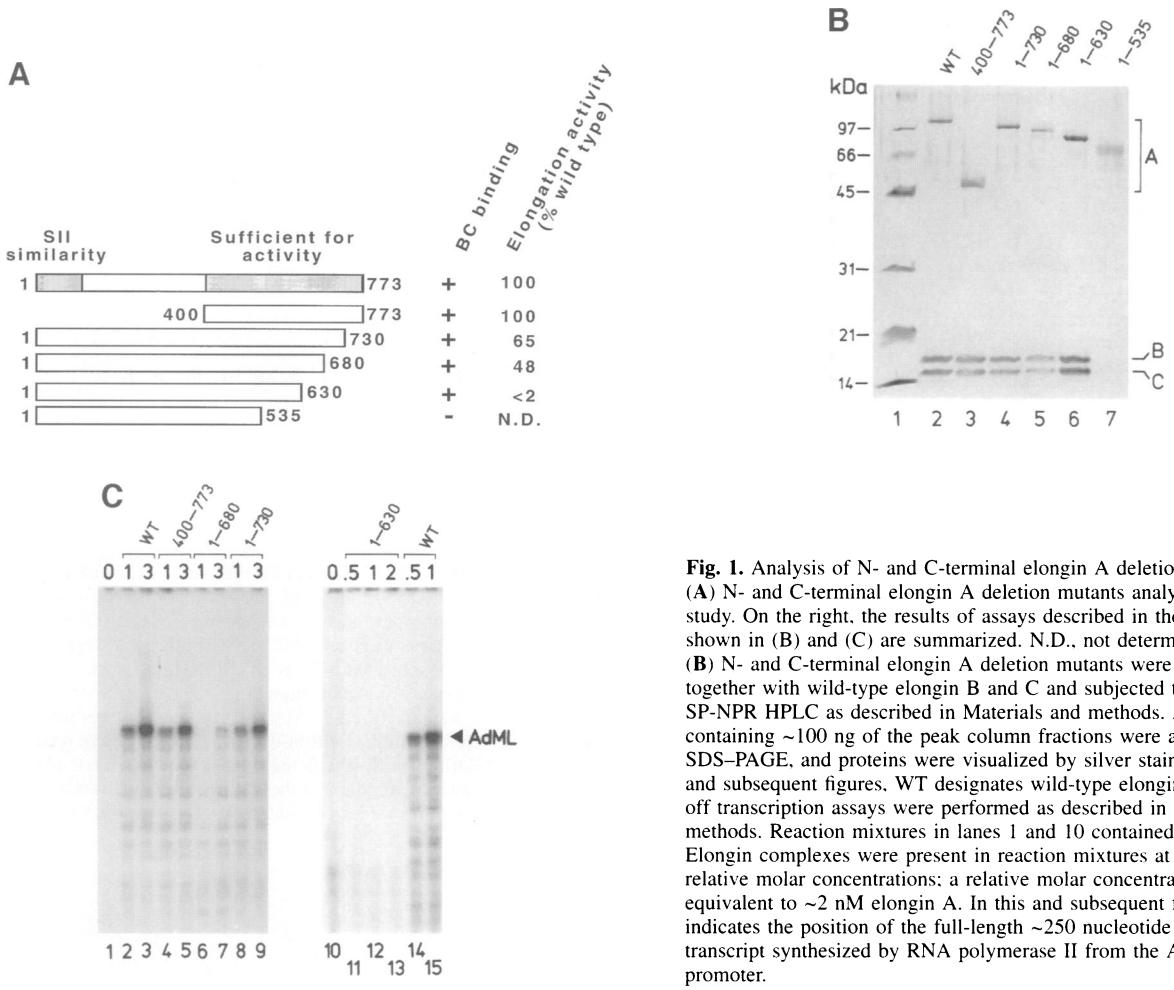
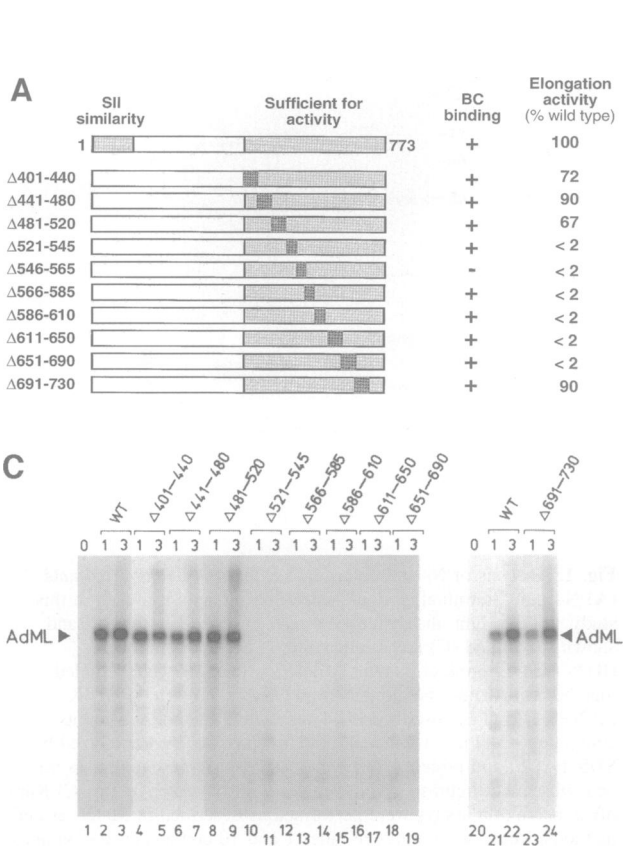



**Fig. 1.** Analysis of N- and C-terminal elongin A deletion mutants. (**A**) N- and C-terminal elongin A deletion mutants analyzed in this study. On the right, the results of assays described in the text and shown in (B) and (C) are summarized. N.D., not determined. (**B**) N- and C-terminal elongin A deletion mutants were refolded together with wild-type elongin B and C and subjected to TSK SP-NPR HPLC as described in Materials and methods. Aliquots containing ~100 ng of the peak column fractions were analyzed by SDS–PAGE, and proteins were visualized by silver staining. In this and subsequent figures, WT designates wild-type elongin A. (**C**) Run-off transcription assays were performed as described in Materials and methods. Reaction mixtures in lanes 1 and 10 contained no elongin. Elongin complexes were present in reaction mixtures at the indicated relative molar concentrations; a relative molar concentration of 1 is equivalent to ~2 nM elongin A. In this and subsequent figures, AdML indicates the position of the full-length ~250 nucleotide run-off transcript synthesized by RNA polymerase II from the AdML promoter.

mutations spanning the elongation activation domain from residues 400 to 730 were constructed (Figure 2A), expressed in *E.coli*, purified and assayed as described above. All elongin A internal deletion mutants, except Δ546–565, which lacks the region similar to the elongin BC binding site in the VHL protein (Kibel *et al.*, 1995), were capable of binding to elongin B and C (Figure 2B). As shown in Figure 2C, elongin ABC complexes containing elongin A internal deletion mutants Δ401–440, Δ441–480, Δ481–520 and Δ691–730 all exhibited significant transcriptional activity. In contrast, elongin ABC complexes containing elongin A internal deletion mutants lacking sequences between 521 and 690 were profoundly impaired in their abilities to stimulate elongation by RNA polymerase II. Notably, the elongin ABC complex containing elongin A internal deletion mutant Δ521–545, which lacks the potential topoisomerase I catalytic site motif, is inactive in transcription. Topoisomerase I activity is unlikely to play a role in elongin (SIII) function *in vitro*, however, because (i) we observe that mutation of the potential active site tyrosine at residue 543 to either phenylalanine or serine has no effect on elongin A transcriptional activity (data not shown) and (ii) we have been unable to detect topoisomerase I activity associated with elongin A. Taken together, analysis of the N-terminal, C-terminal and internal elongin A deletion mutants localized sequences critical for transcriptional activity to a minimal region of ~170 amino acids between residues 521 and 690. Further investigation revealed, however, that elongin A sequences outside this minimal region also make significant contributions to elongin A transcriptional activity since elongin A mutants composed of residues 500–730 or residues 500–700 were transcriptionally inactive, even though they assembled into isolable elongin ABC complexes (Figure 3). Thus, elongin A sequences outside the minimal elongation activation domain make secondary contributions to elongin activity, either by participating directly in interactions with the RNA polymerase II elongation complex or by helping to maintain the proper three-dimensional structure of the elongation activation domain.

### *A conserved elongin BC binding site motif shared by elongin A and the VHL tumor suppressor protein*

As described above, analysis of elongin A deletion mutants revealed that the elongin A region between residues 546 and 565 plays an important role in binding to elongin B and C. As shown in Figure 4A, this elongin A region shares sequence similarity with a short region of the VHL protein (residues 157–172, underlined) previously shown to be sufficient for binding to elongin B and C (Kibel *et al.*, 1995). To define this elongin A region further, a systematic series of elongin A internal deletion mutants spanning residues 545–568 were constructed (Figure 4A), expressed in *E.coli*, purified and assayed for their abilities



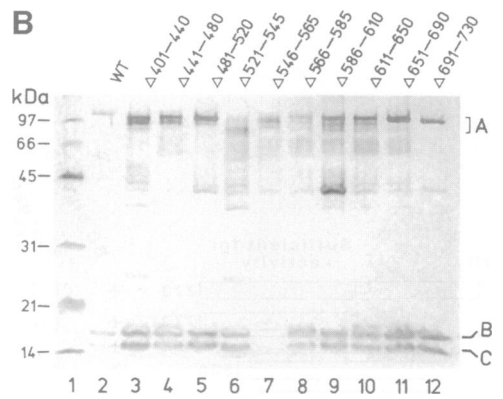




**Fig. 2.** Analysis of elongin A internal deletion mutants. (**A**) Elongin A internal deletion mutants. On the right, the results of assays described in the text and shown in (B) and (C) are summarized. (**B**) Elongin A internal deletion mutants were refolded together with wild-type elongin B and C and subjected to TSK SP-NPR HPLC as described in Materials and methods. Aliquots containing ~100 ng of the peak column fractions were analyzed by SDS–PAGE, and proteins were visualized by silver staining. (**C**) Run-off transcription assays were performed as described in Materials and methods. Elongin complexes were present in reaction mixtures at the indicated relative molar concentrations; a relative molar concentration of 1 is equivalent to ~2 nM elongin A.

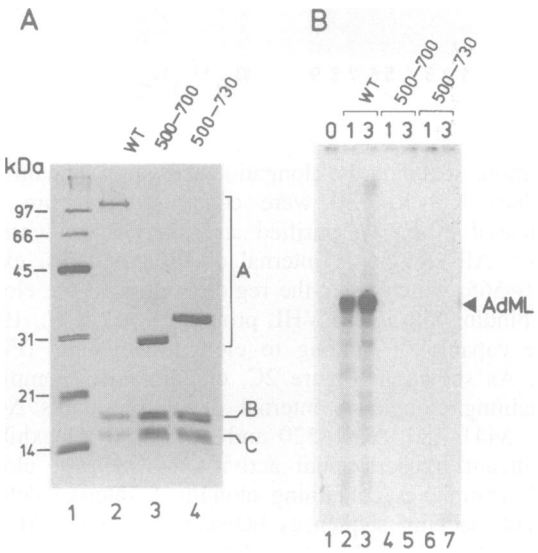
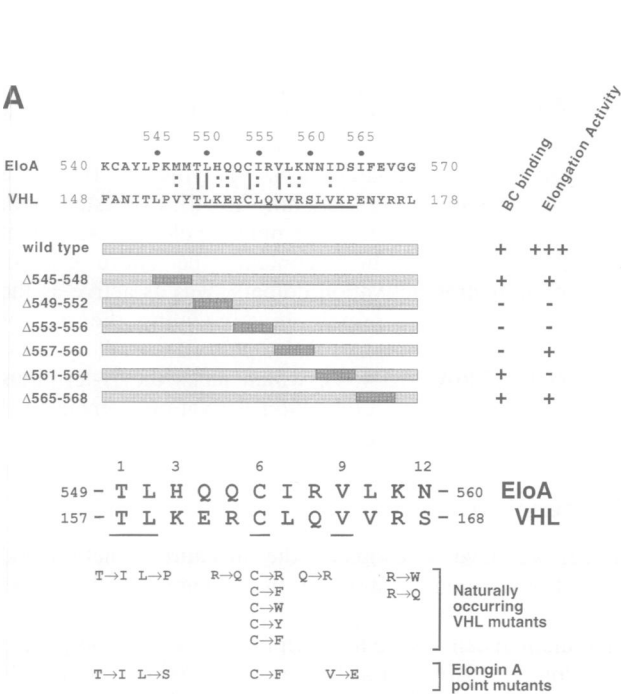



**Fig. 3.** Analysis of short elongin deletion mutants containing the minimal elongation activation domain. (**A**) Elongin A deletion mutants were refolded together with wild-type elongin B and C and subjected to TSK SP-NPR HPLC as described in Materials and methods. Aliquots containing ~100 ng of the peak column fractions were analyzed by SDS–PAGE, and proteins were visualized by silver staining. (**B**) Run-off transcription assays were performed as described in Materials and methods. The reaction mixture in lane 1 contained no elongin. Elongin complexes were present in reaction mixtures at the indicated relative molar concentrations; a relative molar concentration of 1 is equivalent to ~2 nM.

to assemble into chromatographically isolable elongin ABC complexes and to stimulate the rate of elongation by RNA polymerase II.

As shown in Figure 4B, elongin A internal deletion mutants Δ545–548, Δ561–564 and Δ565–568 bound stably to elongin B and C to form elongin ABC complexes that could be purified by TSK SP-NPR HPLC. In contrast, elongin A internal deletion mutants Δ549–552, Δ553–556 and Δ557–560, which each lack elongin A sequences within the region most similar to the elongin BC binding site in the VHL protein, were impaired in their abilities to bind to elongin B and C.

To assess the transcriptional activities of these elongin A internal deletion mutants, aliquots of peak fractions shown in Figure 4B from TSK SP-NPR HPLC purification of mutant elongin ABC complexes were assayed for their abilities to stimulate the rate of elongation by RNA polymerase II. Because they co-purified with variable amounts of elongin B and C, these mutants were assayed in the absence (Figure 5A) and presence (Figure 5B) of excess purified elongin BC complex. The results of these assays revealed that all elongin A deletion mutants with mutations between residues 545 and 568 were transcriptionally impaired, although not all mutants were impaired to the same extent. Two elongin A deletion mutants, Δ545–548 and Δ565–568, which efficiently formed isolable elongin ABC complexes, exhibited reduced but readily detectable transcriptional activity, whereas elongin A mutant Δ561–564, which could also bind elongin B and C, exhibited little activity. In addition, two elongin A deletion mutants, Δ549–552 and Δ553–556, which were severely impaired in their abilities to form isolable elongin ABC complexes, exhibited little or no detectable transcriptional activity, whereas elongin A mutant Δ557–560, which was also impaired in its ability to form an isolable elongin ABC complex, exhibited detectable activity, raising the



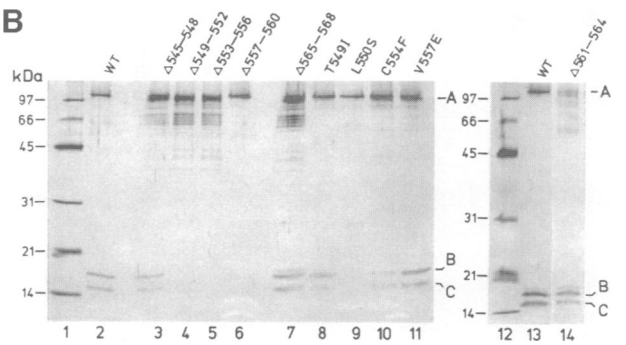




**Fig. 4.** Analysis of elongin A internal deletion and point mutants with mutations in the potential elongin BC binding site. (**A**) Elongin A internal deletion mutants. At the top is shown a comparison of the similar regions of elongin A and the VHL protein. The portion of the VHL protein shown to be sufficient for binding to elongin B and C (Kibel *et al.*, 1995) is underlined; vertical lines indicate identical amino acids; colons indicate chemically similar amino acids. EloA, elongin A; VHL, VHL protein. At the bottom are shown the synthetic elongin A point mutants and a summary of naturally occurring VHL mutants. (**B**) Elongin A internal deletion mutants were refolded together with wild-type elongin B and C and subjected to TSK SP-NPR HPLC as described in Materials and Methods. Aliquots containing ~100 ng of the peak column fractions were analyzed by SDS–PAGE, and proteins were visualized by silver staining.

possibility that deletion of elongin A residues between 557 and 560 may result in an increase in the basal activity of elongin A.

The results described above indicate that elongin A sequences most critical for binding to elongin B and C fall within a 12 amino acid region between residues 549 and 560. Both elongin A and the VHL protein share conserved threonine, leucine, cysteine and valine residues in this region at the positions designated 1, 2, 6 and 9 at the bottom of Figure 4A. Investigations of mutations found in VHL families and in clear-cell renal carcinoma have identified naturally occurring VHL point mutations of the conserved threonine, leucine and cysteine residues, but not of the conserved valine residue (Latif *et al.*, 1993; Foster *et al.*, 1994; Gnarra *et al.*, 1994; Kanno *et al.*, 1994; Whaley *et al.*, 1994; Chen *et al.*, 1995). To investigate the importance of these residues for elongin A transcriptional activity, elongin A mutants carrying point mutations of the conserved threonine, leucine, cysteine and valine residues were constructed, expressed in *E.coli*, purified and assayed for their abilities to form isolable elongin ABC complexes and to stimulate the rate of elongation by RNA polymerase II. As shown in Figure 4B, each of the elongin A point mutants, except L550S, formed readily detectable elongin ABC complexes that could be purified by TSK SP-NPR HPLC, although elongin A mutants T549I and C554F appeared somewhat impaired in their abilities to form elongin ABC complexes.

To assess the transcriptional activities of the elongin A point mutants, aliquots of peak fractions shown in Figure 4B were assayed for their abilities to stimulate the rate of elongation by RNA polymerase II in the absence (Figure 5A) and presence (Figure 5B) of excess purified elongin BC complex. Interestingly, only elongin A point mutant V557E, which is mutated at a position where there are no corresponding naturally occurring VHL mutations, exhibited near wild-type activity.

### *Evolutionary conservation of the elongin A elongation activation domain*

A TBLASTN search of the GenBank non-redundant database using rat elongin A as the query sequence identified a predicted *C.elegans* ORF encoding a potential elongin A homolog. By screening a *C.elegans* cDNA library with a probe derived from the predicted ORF, we isolated a cDNA encoding a highly basic, 434 amino acid protein with a calculated $M_r$ of 49.2 kDa. Comparison of the predicted amino acid sequences of mammalian elongin A and the potential *C.elegans* elongin A homolog revealed two conserved regions: an N-terminal region resembling the SII-like N-terminus of mammalian elongin A and a C-terminal region resembling the C-terminal elongation activation domain of mammalian elongin A. Notably, the potential *C.elegans* elongin A homolog exhibited the greatest similarity (33% identity, 53% similarity, alignment score 17.8 SD) to mammalian elongin A residues 520–662, which include the majority of the region most critical for transcriptional activity (Figure 6). In addition, this region of the *C.elegans* protein includes a short sequence that resembles the elongin BC binding site in the VHL protein, but it lacks the topoisomerase I catalytic site motif.

To determine the functional relationship between mammalian elongin A and the potential *C.elegans* elongin A homolog, the intact *C.elegans* ORF and two N-terminal deletion mutants C.e.EloA(94–434) and C.e.EloA(202–434) were constructed, expressed in *E.coli*, purified and assayed for their abilities to interact with mammalian elongin B and C and to stimulate the rate of elongation by mammalian RNA polymerase II. Mutant C.e.EloA(94–434) lacks sequences related to SII, but contains sequences similar to the elongin A elongation activation domain and elongin BC binding site. Mutant C.e.EloA(202–434) contains the potential elongin BC binding site, but lacks sequences similar to the N-terminus of the elongation activation domain of mammalian elongin A.

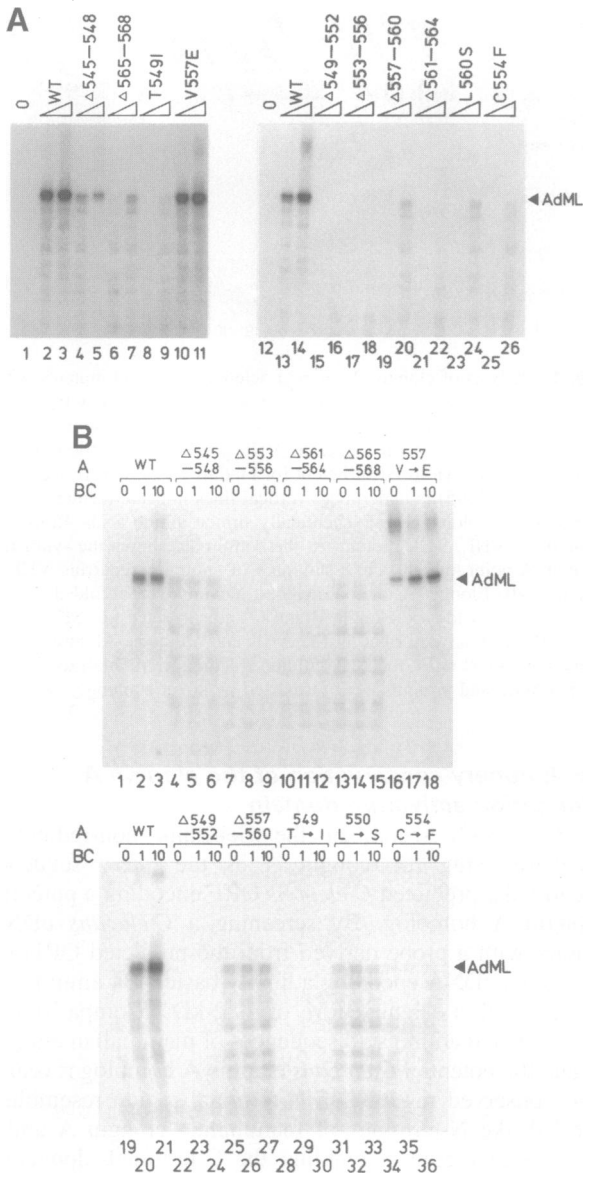



**Fig. 5.** Transcription activity of elongin A internal deletion and point mutants with mutations in the potential elongin BC binding site. (**A**) Run-off transcription assays were performed as described in Materials and methods. The concentration of elongin complexes was adjusted so that elongin A was present in reaction mixtures at either 2 or 6 nM. (**B**) Run-off transcription assays were performed as described in Materials and methods in the absence and presence of excess wild-type elongin BC. Run-off transcription assays were performed in the presence of either 4 nM TSK SP-NPR-purified wild-type elongin A or 4 nM TSK SP-NPR-purified mutant elongin complexes and the indicated relative molar concentrations of purified elongin BC complex; a relative molar concentration of 1 is equivalent to ~4 nM elongin BC. BC, purified elongin BC complex.

Both the full-length *C.elegans* protein and mutants C.e.EloA(94–434) and C.e.EloA(202–434) were capable of binding to mammalian elongin B and C (Figure 7A and data not shown). In addition, the full-length *C.elegans* protein was capable of stimulating the rate of elongation by mammalian RNA polymerase II in a reaction dependent on mammalian elongin B and C (Figure 7B), although it is less active than mammalian elongin A. In the experiment shown, the transcriptional activities of mammalian elongin A and the *C.elegans* protein were compared using an oligo(dC)-tailed template assay (Kadesch and Chamberlin, 1982; Tan *et al.*, 1994a), which permits direct measurement of the effect of elongation factors on the rate of RNA chain elongation by RNA polymerase II in the absence of initiation factors. *C.elegans* elongin A also stimulates elongation by mammalian RNA polymerase II in the promoter-specific assay (data not shown). Consistent with our results from analysis of mammalian elongin A, mutant C.e.EloA(94–434), which contains the entire elongin A-like elongation activation domain, was as active as the wild-type *C.elegans* protein in stimulating the rate of elongation by mammalian RNA polymerase II, whereas mutant C.e.EloA(202–434), which lacks the N-terminus of the elongin A-like elongation activation domain, had significantly reduced activity.

## Discussion

Here, we have investigated the structure, function and evolutionary conservation of the elongin A elongation activation domain. Elongin A was initially purified from mammalian cells as the transcriptionally active component of elongin (SIII) (Bradsher *et al.*, 1993a; Aso *et al.*, 1995b), a multimeric complex composed of A, B and C subunits of 773, 118 and 112 amino acids. Biochemical studies have shown that elongin B and C are regulatory subunits, which bind stably to each other to form a binary complex that assembles with elongin A to form the intact elongin (SIII) complex and, in the process, strongly induces elongin A transcriptional activity (Aso *et al.*, 1995b; Duan *et al.*, 1995b; Garrett *et al.*, 1995).

### *The elongin A elongation activation domain*

As part of our effort to understand how the activity of elongin A is regulated by the elongin BC complex, we have carried out a systematic structure–function analysis of elongin A. Preliminary studies revealed that an elongin A region encompassing amino acids 400–773 includes all sequences necessary for full elongin A transcriptional activity and for regulation by elongin BC. Further investigation localized the most critical sequences within the elongin A elongation activation domain to an ~160 amino acid region between residues 521 and 680. An elongin A mutant composed of residues 500–730 was inactive, however, suggesting that elongin A sequences outside this region, though not essential for transcription, either participate in interactions with the RNA polymerase II elongation complex or help to maintain the structural integrity of the elongation activation domain.

Previously characterized transcriptional activation domains fall into several broad classes according to their amino acid compositions. These include acidic activators such as VP16, proline-rich activators such as CTF/NF1, serine- and threonine-rich activators such as Pit-1, and glutamine-rich activators such as Sp1 (Mitchell and Tjian, 1989; Ingraham *et al.*, 1990). These activators previously were thought to function exclusively in the regulation of initiation; however, recent studies indicate that at least some of them can also regulate the efficiency of elongation by RNA polymerase II (Yankulov *et al.*, 1994; Krumm *et al.*, 1995). Although elongin A contains a conspicuous cluster of acidic amino acids between residues 388 and 410, a serine- and threonine-rich region between residues

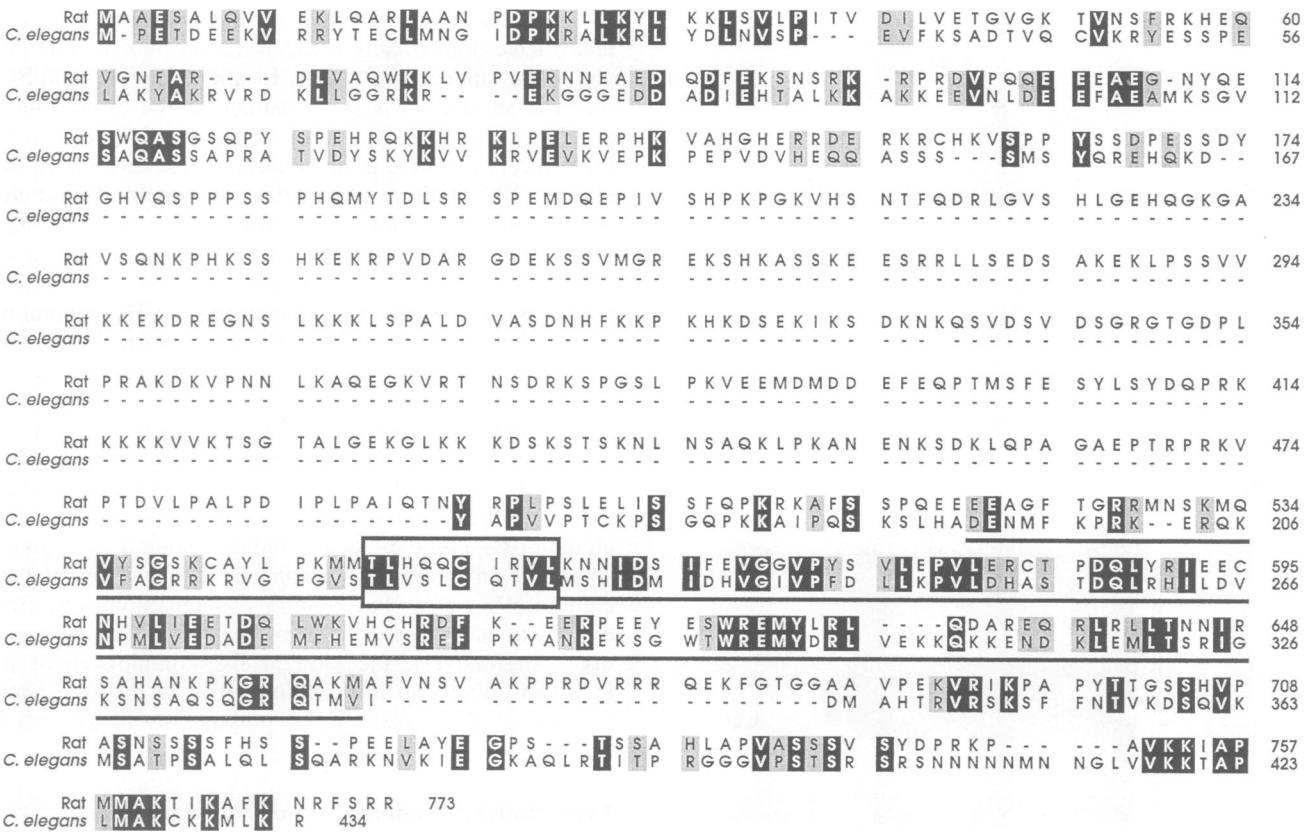



**Fig. 6.** Comparison of the amino acid sequences of rat and *C.elegans* elongin A. Identical amino acids are shaded with black; chemically similar amino acids are shaded with gray. The elongin BC binding site motif is boxed, and the region most highly conserved between rat and *C.elegans* elongin A is underlined.

704 and 744 and a proline-rich region between residues 463 and 498, each of these regions can be deleted without dramatically affecting elongin A transcriptional activity. In addition, based on the results of both homology and MOTIFs searches, the elongin A elongation activation domain lacks structures, such as zinc fingers or helix–turn–helix motifs, which are commonly found in transcriptional activators. Thus, the elongin A elongation activation domain appears to define a structurally distinct type of activator that targets the RNA polymerase II elongation complex.

We noted previously that the N-terminus of elongin A shares significant sequence similarity with the N-terminus of elongation factor SII (Aso *et al.*, 1995b). The results described here indicate that the N-terminus of elongin A is dispensable for stimulation of elongation by RNA polymerase II *in vitro*. Likewise, the N-terminus of SII has been shown to be dispensable for its transcriptional activity *in vitro* and for rescue of the phenotype of an SII deletion mutant in yeast (Marshall *et al.*, 1990; Reines, 1992; Cipres-Palacin and Kane, 1994; Nakanishi *et al.*, 1995). The striking similarity between the N-termini of elongin A and SII, however, suggests that this region may have some function not yet revealed by current assays.

Elongin A also contains a potential ATP binding site (Walker *et al.*, 1982; Gorbalenya *et al.*, 1989) and a near consensus topoisomerase I catalytic site (Eng *et al.*, 1989; Lynn *et al.*, 1989). The potential ATP binding site is located within the SII-like region at the N-terminus of elongin A and is therefore not required for transcriptional activity. The potential topoisomerase I catalytic site is located in an elongin A region (residues 530–549) that is required for transcription. Topoisomerase I activity is unlikely to be required for elongin A transcriptional activity, however, because mutation of the potential active site tyrosine at residue 543 to either phenylalanine or serine has no effect on elongin A transcriptional activity.

### *A conserved elongin BC binding site motif in elongin A and the VHL tumor suppressor protein*

Although both elongin A and the VHL tumor suppressor protein bind elongin B and C with high affinity, elongin A and the VHL protein share only a short region of similarity between residues 547 and 559 of elongin A and residues 155 and 167 of the VHL protein (Aso *et al.*, 1995b). This region of the VHL protein frequently is mutated in VHL kindreds and in sporadic clear-cell renal carcinoma (Latif *et al.*, 1993; Foster *et al.*, 1994; Gnarra *et al.*, 1994; Kanno *et al.*, 1994; Whaley *et al.*, 1994; Chen *et al.*, 1995), and a number of these VHL mutants have been tested and found to be impaired in their abilities to bind elongin B and C (Gnarra *et al.*, 1994; Duan *et al.*, 1995b; Kibel *et al.*, 1995). In addition, a peptide replica of the wild-type VHL sequence between residues 157 and 172 was found to block the interaction of VHL with elongin B and C, whereas a peptide containing a point mutation corresponding to a naturally occurring VHL mutant was not (Kibel *et al.*, 1995). Finally, a GST–VHL fusion protein containing VHL residues 157–172 is capable of binding specifically to elongin B and C (Kibel *et al.*,

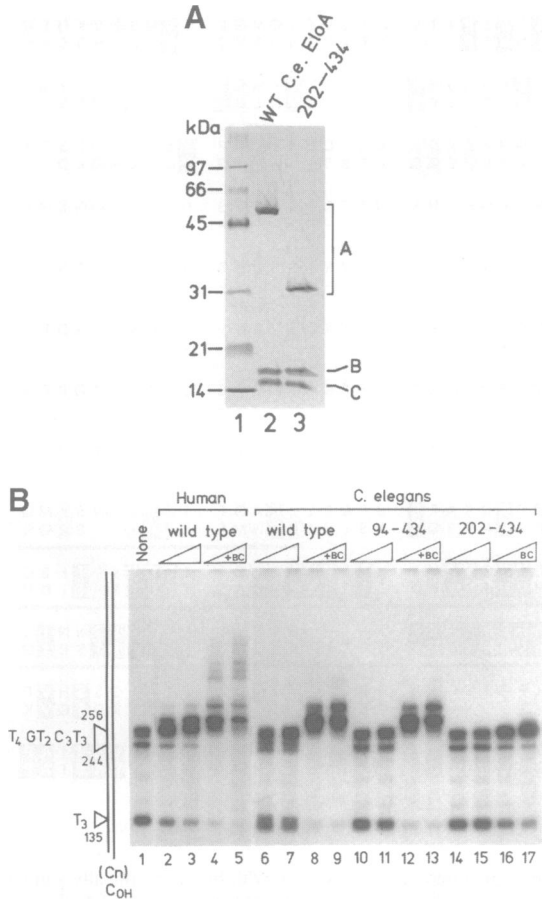



**Fig. 7.** Analysis of wild-type and mutant *C.elegans* elongin A. (**A**) Wild-type or mutant *C.elegans* elongin A was refolded with rat elongin B and C and subjected to TSK SP-NPR HPLC as described in Materials and methods. Aliquots of the peak column fractions were analyzed by SDS–PAGE, and proteins were visualized by silver staining. WT C.e. EloA, wild type *C.elegans* elongin A; 202–434, *C.elegans* elongin A mutant containing residues 202–434. (**B**) Approximately 2 pmol of human (Aso *et al.*, 1996) or *C.elegans* elongin A proteins were refolded as described (Bradsher *et al.*, 1993a) in the presence or absence of ~2 pmol of rat elongin B and C. Aliquots of refolded protein were assayed for transcriptional activity in oligo(dC)-tailed template assays performed as described in Materials and methods. Reaction mixtures contained no elongin (lane 1); 3 and 9 μl of renatured human elongin A without (lanes 2 and 3) or with (lanes 4 and 5) elongin B and C; 3 and 9 μl of renatured wild-type *C.elegans* elongin A without (lanes 6 and 7) or with (lanes 8 and 9) elongin B and C; 3 and 9 μl of renatured C.e. EloA(94–434) without (lanes 10 and 11) or with (lanes 12 and 13) elongin B and C; and 3 and 9 μl of renatured C.e. EloA (202–434) without (lanes 14 and 15) or with (lanes 16 and 17) elongin B and C.

1995), arguing strongly that these residues of the VHL protein comprise an elongin BC binding site.

Results of our mutational analysis indicate that elongin A sequences most critical for binding elongin B and C fall within the 12 amino acid region between residues 549 and 560. Within this conserved elongin BC binding site motif [consensus sequence TLx3Cx2V(V,L)], identical threonine, leucine, cysteine and valine residues are found in elongin A and VHL at the positions designated 1, 2, 6 and 9 at the bottom of Figure 4A. Previous studies have identified naturally occurring VHL mutations in this region at positions 1, 2 and 6, but not at position 9 (Latif *et al.*, 1993; Foster *et al.*, 1994; Gnarra *et al.*, 1994; Kanno *et al.*, 1994; Whaley *et al.*, 1994; Chen *et al.*, 1995). Notably, elongin A point mutants altered at positions 1, 2 and 6 have dramatically reduced transcriptional activities and are not induced detectably by addition of elongin BC, whereas an elongin A point mutant altered at position 9 is fully active. While there is a strong correlation between the effects of mutating the conserved residues within the elongin BC binding site motifs of elongin A and the VHL protein, it is noteworthy that at least two naturally occurring VHL mutations change non-conserved residues at positions 5 and 8 to residues found at the corresponding positions in elongin A. Whether these VHL mutations affect the interaction of the VHL protein with elongin BC or whether they affect a different, as yet unknown, function of the VHL protein is not clear.

Although these studies have defined elongin A sequences important for induction of elongin A transcriptional activity by elongin BC, it has not yet been established at the molecular level how this induction occurs. The elongin BC complex could induce elongin A activity solely by an allosteric mechanism, in which binding of elongin BC causes the elongin A elongation activation domain to adopt a more transcriptionally active conformation. Alternatively, the elongin BC complex could be involved directly in interactions with one or more components of the ternary RNA polymerase II elongation complex.

### *Evolutionary conservation of the elongin A elongation activation domain*

In an effort to identify model systems with which to study the function of elongin (SIII) *in vivo*, we are identifying and characterizing homologs of the elongin subunits in genetically tractable organisms. A homology search of the GenBank database identified a *C.elegans* gene that encodes a 434 amino acid protein that includes regions exhibiting sequence similarity to the N-terminal SII-like region and the C-terminal elongation activation domain of mammalian elongin A, but lacks sequences similar to the region between residues ~170 and 490 of the mammalian protein. As we have shown, the potential *C.elegans* elongin A homolog binds mammalian elongin B and C and is capable of stimulating elongation by mammalian RNA polymerase II. Importantly, the most highly conserved region of the *C.elegans* protein corresponds to the elongin A region identified in our assays as most critical for elongin A transcriptional activity. Like the elongin A elongation activation domain, this region includes a potential elongin BC binding site motif at its N-terminus. It is noteworthy that the *C.elegans* protein conserves neither the potential ATP binding site nor the potential topoisomerase I catalytic site, consistent with our observation that these regions of mammalian elongin A are not essential for transcriptional activity *in vitro*. The identification of a structural and functional *C.elegans* homolog of elongin A should provide a foundation for future genetic investigations into the role of elongin A and the elongin (SIII) complex *in vivo* in the growth and development of a multicellular organism.

## Materials and methods

### *Materials*

Unlabeled ultrapure ribonucleoside 5′-triphosphates were purchased from Pharmacia Biotech Inc. [α-$^{32}$P]CTP (>400 Ci/mmol) was obtained from

Amersham Corp. Phenylmethylsulfonyl fluoride (PMSF), heparin and polyvinyl alcohol type II were from Sigma. Bovine serum albumin (BSA; Pentex fraction V) was purchased from ICN ImmunoBiologicals. Glycerol (Spectranalyzed grade) and guanidine hydrochloride (electrophoresis grade) were obtained from Fisher.

### Expression and purification of wild-type and mutant rat elongin A

Overexpression of rat elongin A mutants in *E.coli* was accomplished using an M13mpET bacteriophage expression system (Tan *et al.*, 1994b). Constructs for expression of 6-histidine-tagged N- and C-terminal elongin A deletion mutants were prepared by inserting PCR-generated fragments of the elongin A cDNA into the *Sal*I and *Bam*HI sites of M13mpET. Constructs for expression of elongin A internal deletion and point mutants were prepared by oligonucleotide-directed mutagenesis (Kunkel, 1985) using the Muta-Gene M13 *in vitro* mutagenesis kit (Bio-Rad). Elongin A mutants were sequenced by the dideoxy chain termination method using a Sequenase kit (United States Biochemicals).

To prepare elongin A mutants, 500 ml cultures of *E.coli* strain JM109(DE3) were grown to an $OD_{600}$ of 0.6 in Luria broth containing 2.5 mM $MgCl_2$ at 37°C with gentle shaking. Cells were infected with M13mpET vectors carrying mutant elongin A cDNAs at a multiplicity of infection of 20. After an additional 2 h at 37°C, cells were induced with 0.4 mM isopropyl β-D-thiogalactoside, and cultures were incubated for an additional 2.5 h. Cells were harvested by centrifugation at 2000 *g* for 10 min at 4°C. The cell pellets were resuspended in 25 ml of ice-cold 20 mM Tris–HCl (pH 8.0), 10 mM imidazole (pH 8.0) and 1 mg/ml lysozyme and incubated on ice for 30 min. After two cycles of freeze–thaw, the suspension was centrifuged at 100 000 *g* for 35 min at 4°C. Inclusion bodies were solubilized by resuspension in 25 ml of ice-cold 50 mM Tris–HCl (pH 8.0) containing 6 M guanidine hydrochloride, and the resulting suspension was clarified by centrifugation at 50 000 *g* for 20 min at 4°C. Recombinant elongin A mutants were purified from the supernatants by $Ni^{2+}$-nitrilotriacetic acid (NTA)–agarose (Invitrogen) affinity chromatography as described (Garrett *et al.*, 1994; Aso *et al.*, 1995b).

### Reconstitution and purification of elongin complexes containing elongin A mutants

Recombinant elongin B and C were expressed in *E.coli* using the M13mpET bacteriophage expression system and purified as described previously (Garrett *et al.*, 1994, 1995). Reconstitution of elongin complexes was carried out essentially as described (Aso *et al.*, 1995b) by refolding ~50 μg of wild-type or mutant elongin A, ~8 μg of elongin B and ~8 μg of elongin C. 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×4.6 mm, Hewlett-Packard) equilibrated in 40 mM HEPES–NaOH (pH 7.9), 0.1 mM EDTA, 1 mM dithiothreitol (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 0.6 ml/min with a 9 ml linear gradient from 0.1 to 0.8 M KCl in the same buffer. Aliquots of each column fraction were analyzed by 12% SDS–PAGE, and the proteins were visualized by silver staining.

### Preparation of RNA polymerase II and initiation factors

RNA polymerase II (Conaway and Conaway, 1990) and TFIIH (rat δ, TSK DEAE 5-PW fraction) (Conaway *et al.*, 1992) were purified as described from rat liver nuclear extracts. Recombinant yeast TATA binding protein (TBP; Conaway *et al.*, 1991) and rat TFIIB (rat α) (Tsuboi *et al.*, 1992) were expressed in *E.coli* and purified as described. Recombinant TFIIE was prepared as described (Peterson *et al.*, 1991), except that the 56 kDa subunit was expressed in BL21(DE3)-pLysS. Recombinant TFIIF was purified as described (Tan *et al.*, 1994b) from *E.coli* strain JM109(DE3) infected with M13mpET-RAP30 and M13mpET-RAP74.

### Assay of run-off transcription

Unless indicated otherwise, pre-initiation complexes were assembled as described (Garrett *et al.*, 1995) by pre-incubation of 50 ng of the *Eco*RI–*Nde*I fragment from pDN-AdML (Conaway and Conaway, 1988) and ~10 ng of recombinant TFIIB, 10 ng of recombinant TFIIF, 7 ng of recombinant TFIIE, 40 ng of TFIIH (rat δ, fractionVI), 50 ng of recombinant yeast TBP (AcA 44 fraction) and 0.01 U of RNA polymerase II. Transcription was initiated by addition of 7 mM $MgCl_2$, 50 μM ATP, 2 μM UTP, 10 μM CTP, 50 μM GTP and 10 μCi [α-$^{32}$P]CTP, either in the absence or presence of elongin preparations. After an 8 min incubation at 28°C, run-off transcripts were analyzed by electrophoresis through 6% polyacrylamide–7.0 M urea gels. Transcription was quantitated using a Molecular Dynamics phosphorimager.

### Oligo(dC)-tailed template assay of elongation by RNA polymerase II

Pulse–chase assays were carried out essentially as described (Aso *et al.*, 1995b). RNA polymerase II (0.01 U) and 100 ng of pCpGR220S/P/X were incubated at 28°C in the presence of 20 mM HEPES–NaOH, pH 7.9, 20 mM Tris–HCl, pH 7.9, 2% (w/v) polyvinyl alcohol, 0.5 mg/ml BSA, 60 mM KCl, 50 μM $ZnSO_4$, 7 mM $MgCl_2$, 0.2 mM DTT, 3% (v/v) glycerol, 3 U of recombinant RNasin (Promega), 50 μM ATP, 50 μM GTP, 2 μM CTP and 10 μCi of [α-$^{32}$P]CTP. After 25 min labeling, the reactions were chased for 7.5 min following addition of 100 μM non-radioactive CTP, 2 μM UTP and the indicated amounts of elongin preparations. Transcripts were analyzed by electrophoresis through 6% polyacrylamide–7.0 M urea gels.

### Isolation of cDNA encoding C.elegans elongin A

A homology search of the GeneBank database using rat elongin A protein sequence as the query revealed that nucleotides 19 335–21 154 of *C.elegans* cosmid R03D7 contained a predicted ORF encoding a protein highly homologous to portions of elongin A. A λ ZAP *C.elegans* cDNA library was constructed and screened with the 5′-$^{32}$P-labeled oligonucleotide 5′-GAGTTGGTCA GTGCTCGCGT GGTCAAGAAC AGGCTTCAGT AGATCAAATGG-3′, which corresponds to a portion of the predicted ORF. Hybridization was performed at 65°C for 20 h in 5× standard saline citrate, 5× Denhardt's solution, 100 mM sodium phosphate, 0.1% sodium dodecylsulfate and 10% dextran sulfate containing denatured salmon testis DNA (100 μg/ml). Three overlapping cDNA clones with inserts of up to 1.8 kb were isolated. Clone CE22, which contained the longest insert, was sequenced on both strands by the dideoxy chain termination method using a Sequenase kit (United States Biochemicals).

### Expression and purification of C.elegans elongin A

Histidine-tagged *C.elegans* elongin A was overexpressed in *E.coli* using a pET16b expression vector (Novagen). The pET16b constructs for expression of histidine-tagged wild-type and mutant *C.elegans* elongin A were prepared by insertion of PCR-generated fragments of the *C.elegans* ORF into the *Nde*I and *Bam*HI sites of the pET16b vector. A 100 ml culture of *E.coli* strain BL21(DE3) transformed with pET16b carrying the *C.elegans* elongin A cDNA was grown to an $OD_{600}$ of 0.6 in Luria broth containing 50 μg/ml ampicillin at 37°C. Following induction with 1 mM isopropyl β-D-thiogalactoside, the culture was incubated for an additional 2.5 h at 37°C. Cells were harvested by centrifugation at 2000 *g* for 10 min at 4°C. The cell pellet was resuspended in 5 ml of ice-cold 20 mM Tris–HCl (pH 8.0), 10 mM imidazole (pH 8.0) and 1 mg/ml lysozyme and incubated on ice for 30 min. After two cycles of freeze–thaw, the suspension was centrifuged at 100 000 *g* for 35 min at 4°C. Inclusion bodies were solubilized by resuspension in 5 ml of ice-cold 50 mM Tris–HCl (pH 8.0) containing 6 M guanidine hydrochloride, and the resulting suspension was clarified by centrifugation at 50 000 *g* for 20 min at 4°C. Recombinant wild-type and mutant *C.elegans* elongin A were purified from the supernatants by $Ni^{2+}$-NTA–agarose (Invitrogen) affinity chromatography as described (Garrett *et al.*, 1994).

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