Identification and Biochemical Characterization of a Novel Transcription Elongation Factor, Elongin A3*

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Katsuhisa Yamazaki‡§, Limei Guo¶, Kazunori Sugahara¶, Chun Zhang‡, Hideaki Enzani‡, Yusaku Nakabeppu¶, Shigetaka Kitajima‡, and Teijiro Aso†**

From the ¶Department of Biochemical Genetics, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, the §§Medical Institute of Bioregulation, Kyushu University and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, and the Departments of ¶Chemistry and ¶Pathology, Faculty of Medicine, Kochi Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan.
As part of our effort to understand the function, mechanism of action, and regulation of the Elongin complex, we are attempting to identify members of the Elongin family. In this report, we describe the identification and biochemical characterization of Elongin A3, a novel Elongin A-related RNA polymerase II elongation factor from human cells.

**Experimental Procedures**

**Materials**—Unlabeled ultrapure ribonucleoside 5′-triphosphate and [α-32P]CTP (>400 Ci/nmol) were purchased from Amersham Biosciences. Bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), and antipain (Sigma), and bovine testis RNA, were obtained from Sigma, placental ribonuclease inhibitor from Promega, and NiCl2-nitrirocetic acid agarose (Ni2+-agarose) from Qiagen.

**Isolation of Human Elongin A3 cDNA**—The data base of human expressed sequence tags (ESTs) was searched with the human Elongin A cDNA sequence (accession no. AC011814), and it was revealed that part of the sequence on human chromosome 18 contained this fragment and sequences that were similar to coding regions of both Elongin A and Elongin A2 cDNAs. The full-length human cDNA was isolated from a human placenta cDNA library (Clontech) using an Elongin A3-specific 5′-primer (5′-ATGCCGGAGTTCACTACGCTTG-3′) and 3′-antisense primer (5′-TTATCGCGGAGAATTCTCGTG-3′) using KOD DNA polymerase (TOYOBO).

**DNA Sequencing and Northern Blot Analysis**—DNA sequencing was performed using an automated sequencer (ABI Prism 310, Applied Biosystems). Human multiple tissue Northern blots I and II (CLONTECH) containing 2 and 59 base pair (bp) region cDNA were probed in lysis buffer (50 mM NaCl, 0.1% Nonidet P-40, 0.5 mM PMSF, 5 mM leupeptin, 5 mM aprotinin, and 1 mM benzamidine) and lysed by brief sonication. The cell lysates were clarified by centrifugation for 20 min at 100,000 × g to remove insoluble materials. Recombinant proteins were purified from the soluble fraction of lysates using Ni2+-agarose and anti-FLAG M2-agarose (Sigma) affinity chromatography. Each supernatant (5 ml) was applied to a 3-ml Ni2+-agarose column equilibrated in lysis buffer containing 50 mM imidazole (pH 7.9) and incubated for 30 min at 4°C. The column was then washed with 20 ml of lysis buffer containing 10 mM imidazole (pH 7.9). The recombinant proteins were eluted with 10 ml of lysis buffer containing 300 mM imidazole (pH 7.9), and the peak fractions of each eluate were dialyzed against the IP buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, and 0.1% Nonidet P-40). The dialyzed samples were then analyzed on 7.5% or 12% polyacrylamide, 7.0 M urea gels.

**Expression of Recombinant Proteins in Insect Cells**—Spodoptera frugiperda (Sf9) cells were cultured in Grace’s insect medium (Invitrogen) supplemented with 10% fetal bovine serum (JRH Biosciences) at 27 °C. A recombinant transfer plasmid containing either a wild-type or mutant Elongin A3 cDNA was cotransfected with Bsu36I-digested BacPAK6 virus DNA (CLONTECH) into Sf9 cells by Lipofectamine-mediated (Invitrogen) transfection, and the resultant viral particles were harvested 72 h post-infection. The pellets were resuspended in 1 ml of lysis buffer containing 20 mM imidazole (pH 7.9) and centrifuged for 1 min at 2000 rpm. After resuspending in lysis buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 0.1% Nonidet P-40, 0.5 mM PMSF, 5 μg/ml of leupeptin, 5 μg/ml of aprotinin, and 1 mM benzamidine) and lysed by brief sonication. The cell lysates were clarified by centrifugation for 20 min at 100,000 × g to remove insoluble materials. Recombinant proteins were purified from the soluble fraction of lysates using Ni2+-agarose and anti-FLAG M2-agarose (Sigma) affinity chromatography. Each supernatant (5 ml) was applied to a 3-ml Ni2+-agarose column equilibrated in lysis buffer containing 50 mM imidazole (pH 7.9) and incubated for 30 min at 4°C. The column was then washed with 20 ml of lysis buffer containing 10 mM imidazole (pH 7.9). The recombinant proteins were eluted with 10 ml of lysis buffer containing 300 mM imidazole (pH 7.9), and the peak fractions of each eluate were dialyzed against the IP buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, 0.1% Nonidet P-40, and 200 μg/ml of FLAG peptide). Aliquots of the eluates were used for SDS-PAGE and in vitro transcription elongation assay.

**Oligo(dC)-tailed Template Assay of Transcription Elongation Factor by RNA Polymerase II—**RNA polymerase II was purified as described from rat liver nuclear extracts (21). Pulse-chase assays were carried out essentially as reported previously (9). RNA polymerase II (0.01 units) and 100 ng of pCpGR220S/PX 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 of bovine serum albumin, 60 mM KCl, 50 mM ZnSO4, 7 mM MgCl2, 0.2 mM dithiothreitol, 3% (v/v) glycerol, 3 units of mammalian ribonuclease inhibitor, 50 μM ATP, 50 μM GTP, 2 μM CTP, and 10 μCi [α-32P]CTP. After 20 min of labeling, 100 μM nonradioactive CTP, 2 μM UTP, and specific amounts of Elongin preparations were added, and the reactions were incubated for the times indicated in Figs. 4, 5, and 6. Transcripts were analyzed by electrophoresis through 6% polyacrylamide, 7.0 M urea gels.

**Assay of Runoff Transcription—**Pre-initiation complexes were assembled by pre-incubation of 50 ng of the EcoRI-Ndel fragment from pDN-AdML (9) and 10 ng of TFIIB, 10 ng of TFIIF, 7 ng of TFIIE, 40 ng of TFIH, 50 ng of yeast TBP, and 0.01 units of RNA polymerase II. Transcription was initiated by the addition of 7 mM MgCl2, 50 μM ATP, 2 μM UTP, 10 μM CTP, 50 μM GTP, and 10 μCi [α-32P]CTP either in the absence or presence of purified Elongin A3. After incubation for given periods at 28°C, runoff transcripts were analyzed by electrophoresis through 6% polyacrylamide, 7.0 M urea gels.

**In Vitro Protein–Protein Interaction Assay—**Recombinant baculoviruses expressing human Elongin A3 or rat Elongin A with both histidine- and FLAG epitope tags at their NH2 termini were introduced into Sf9 cells either with or without baculoviruses expressing untagged wild-type or mutant Elongin A3. The cells were harvested 72 h post-infection, the supernatants containing the unbound proteins were collected, and the 50 mM imidazole (pH 7.9) and centrifuged for 1 min at 2000 rpm. After resuspending in lysis buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 0.1% Nonidet P-40, 0.5 mM PMSF, 5 μg/ml of leupeptin, 5 μg/ml of aprotinin, and 1 mM benzamidine), and lysed by brief sonication. The cell lysates were incubated for 2 h at 4°C with ~100 μl of Ni2+-agarose pre-equilibrated in lysis buffer containing 20 mM imidazole (pH 7.9) and then centrifuged for 1 min at 2000 rpm. Following centrifugation, the supernatants containing the unbound proteins were collected, and the Ni2+-agarose was washed four times by resuspension in 1 ml of lysis buffer containing 20 mM imidazole (pH 7.9) and centrifuged for 1 min at 2000 rpm. Finally, bound proteins were eluted with 200 μl of lysis buffer containing 300 mM imidazole (pH 7.9). Aliquots of loaded and bound fractions were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with the appropriate antibody.

**Immunostaining of Cells—**COS7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transiently transfected with the pCl-hEloA3 DNA using SuperFect (Qiagen) according to the manufacturer’s protocol. The cells grown in a chamber slide were fixed by immersion in cold acetone/methanol (1:1) for 5 min. After drying, the slides were incubated for 20 min in blocking solution (1% bovine serum albumin, 0.2% Tween 20, and 6.7% glycerol at 4°C overnight, the cells were incubated with anti-FLAG antibody (1:1000) for 1 h, washed with PBS, and sequentially incubated with fluorescence-labeled anti-mouse immunoglobulin G antibody (1:5000) for 30 min. For staining of nuclei, cells were treated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Nacalai Tesque.

**Results and Discussion**

**Isolation of a cDNA Clone for Human Elongin A3—**To study the possible existence of a novel member of the Elongin protein
family, a database of human expressed sequence tags was searched with the human Elongin A sequence (20) as the query. One expressed sequence tag clone (accession no. AW090822) found in a brain database showed 56% identity to the 5’-end of the coding region of human Elongin A at the amino acid level. Subsequent searches using this fragment as a query identified not only DNA sequences that contained this entire fragment but also sequences with homology to the rest of the coding region of both Elongin A and Elongin A2. The full-length human Elongin A3 cDNA was obtained by PCR amplification of pooled human placenta cDNAs using an Elongin A3-specific 5’-sense primer and 3’-antisense primer. The Elongin A3 cDNA contained an open reading frame encoding a protein of 546 amino acids with a calculated molecular mass of 59,759 Da (Fig. 1). As determined by the MegAlign program of the Lasergene (Madison, WI) system, Elongin A3 is 49 and 81% identical to Elongin A and Elongin A2, respectively. Predicted amino acid sequences of Elongin A3 revealed several notable structural features. First, like Elongins A and A2, the NH2-terminal 110 amino acids of Elongin A3 resembled the NH2 terminus of transcription elongation factor SII (29% identity to human SII) (9, 10, 22). Second, Elongin A3 also possesses the Elongin BC binding sequence (residues 336-347) at its COOH terminus. This 10-amino-acid consensus sequence (T/S/I)LXXCXXV/L/D has been found not only in the Elongin A family but also in VHL, MUF1, Rad7, and the suppressor of cytokine signaling (SOCS) family of proteins (9, 16, 23-25). Third, the COOH-terminal region of Elongin A3 contains the sequence LDGDDGGSV (residues 496-504), which perfectly matches the consensus ATP-binding motif LGXGXXGXXV often found in serine/threonine kinases (26, 27). Expression of Elongin A3 in Various Human Tissues—To examine the tissue distribution of Elongin A3, Northern blots containing poly(A)+ RNA from various human tissues were hybridized with Elongin A3-specific probe. As shown in Fig. 2, the Elongin A3 probe hybridized to a single band of ~5.0 kb and, consistent with previous studies, the Elongin A-specific probe hybridized to two mRNA species of ~5.2 and ~2.8 kb (10, 28). To correct for the amount of RNA loaded in each lane, we measured the intensity of each of the bands on the blot after the hybridization with the actin probe. The results of Northern blot analyses indicate that the Elongin A3 and Elongin A mRNAs are expressed in many of the same tissues, although the expression level of each mRNA varies among tissues. Notably, the highest level of expression of Elongin A3 and Elongin A was observed in skeletal muscle and in testis, respectively. Elongin A3 Forms a Stable Complex with Elongin BC—Because of the high degree of conservation of the putative Elongin BC-binding residues in Elongin A3, we explored Elongin A3 to also interact with Elongins B and C. To investigate this interaction, a DNA fragment containing the open reading frame of Elongin A3 was introduced into the baculovirus expression vector. Recombinant Elongin A3 with hexahistidine and FLAG tags at the NH2 terminus was expressed in insect cells and purified from the soluble fraction of the cell lysates by sequential Ni2+-agarose and anti-FLAG M2-agarose affinity
chromatography. Recombinant Elongin A3 had an apparent molecular mass of 75 kDa (Fig. 3A, left panel), and polyclonal antisera raised against a synthetic peptide corresponding to amino acids 491 to 509 of Elongin A3 recognized this protein (Fig. 3A, right panel).

To assess the ability of Elongin A3 to bind with Elongins B and C to form an Elongin A3-Elongin BC complex, we assayed human Elongin A3 containing hexahistidine and FLAG tags for its ability to retain the untagged Elongins B and C on Ni²⁺/H₁₀₀₀₁-agarose. In this experiment, Elongin A3 was coexpressed with Elongins B and C in Sf9 cells, and cell lysates were subjected to Ni²⁺/H₁₀₀₀₁-agarose chromatography. Bound protein fractions were collected, and equivalent amounts of loaded and bound fractions were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting. As shown in Fig. 3B, untagged Elongins B and C did not bind to Ni²⁺-agarose (lanes 3 and 4), but were retained on the column in the presence of hexahistidine-tagged Elongin A3 (lanes 5 and 6). Thus, as predicted by the sequence homology, Elongin A3 can stably bind to Elongin BC.

Elongin A3 Stimulates Transcription Elongation—In previous studies, we demonstrated that both Elongin A and Elongin A2 are capable of stimulating the rate of RNA chain elongation by RNA polymerase II (9, 10). To investigate whether Elongin A3 is also capable of this, we employed two different assays of transcription elongation, the oligo(dC)-tailed template assay and the AdML runoff transcription assay, and measured the kinetics of accumulation of the long transcripts and the distribution of RNA intermediates in the presence or absence of Elongin A3.

To examine the direct effect of Elongin A3 on the rate of RNA chain elongation by RNA polymerase II in the absence of other transcription factors, an oligo(dC)-tailed template assay was performed. In the experiment shown in Fig. 4A, transcription was initiated by the addition of RNA polymerase II to reaction mixtures containing ATP, GTP, and [γ-³²P]CTP and the pCpGR220 S/P/X template. In this template, the first non-template strand (dT) residues are located 136, 137, and 138 nucleotides from the oligo(dC)-tail; the next run of (dT) residues is located 244 to 256 nucleotides from the oligo(dC)-tail. After a 20-min incubation, transcripts of ~135 nucleotides were synthesized on the T-less cassette of the template. These transcripts were then chased with a limiting concentration of UTP and an excess of nonradioactive CTP in the presence or
In the absence of baculovirus expressing recombinant Elongin A3, a substantial portion of the 135-nucleotide transcripts persisted for at least 10 min after the addition of UTP, and nearly all had been chased into longer transcripts at 20 min (lanes 1–4). In the presence of Elongin A3, RNA chain elongation progressed more rapidly, with nearly all of the 135-nucleotide transcripts disappearing within 5 min after the addition of UTP (lanes 5–8).

The specific activity of Elongin A3 was then compared with that of Elongin A using the same assay. In the experiment shown in Fig. 4B, the 135-nucleotide transcripts (lane 1) were chased for 3 min with a limiting concentration of UTP in the presence or absence of equivalent amounts of purified Elongin A3 or Elongin A. In the presence of Elongin A3, a higher proportion of the 135-nucleotide transcripts had been chased into the 250-nucleotide transcripts than in the presence of Elongin A, suggesting that Elongin A3 possesses slightly more specific activity than Elongin A.

Next, to examine the ability of Elongin A3 to increase the rate of RNA chain elongation by RNA polymerase II in the presence of general initiation factors, an AdML runoff transcription assay was performed. In the experiment shown in Fig. 4C, preinitiation complexes were assembled by preincubation of RNA polymerase II, initiation factors, and DNA template, transcription was carried out for the indicated times (top, in minutes) in the absence (lanes 1–6) or presence of 4 nM purified Elongin A3 (lanes 7–12).
under these conditions the rate of RNA chain elongation is very slow, and full-length runoff transcripts were not detectable even 30 min after the addition of ribonucleoside triphosphates unless Elongin A3 was present (lanes 1 to 6). However, with the addition of Elongin A3 transcripts accumulated more rapidly, with the first full-length runoff transcripts appearing within 18 min (lanes 7 to 12). Elongin BC was present in reaction mixtures at 1.5 nM (lanes 3 and 6) or 3 nM (lanes 4 and 7). C, the oligo(dC)-tailed template assays were performed in the absence (lanes 1 and 8) or presence of 3 nM purified Elongin A3 mutant (lanes 2 and 3) or 3 nM purified Elongin A3-A chimeras (lanes 4–7, 9 and 10). Elongin BC was present in reaction mixtures at 3 nM (lanes 3, 5, 7, and 10).

The results of these experiments indicate that Elongin A3 is a novel RNA polymerase II elongation factor that can function either during promoter-independent transcription on an oligo(dC)-tailed template or during promoter-specific transcription in the presence of general initiation factors.

**The COOH-Terminus of Elongin A Is Required for the Transcriptional Activation by Elongin BC**—In a previous study we demonstrated that the transcriptional activity of Elongin A but not Elongin A2 is triggered by Elongin BC (9, 10, 16). To examine the effect of Elongin BC on the transcriptional activity of Elongin A3, a purified Elongin BC complex at increasing concentrations was preincubated at 4 °C for 30 min in the presence of purified Elongin A3, and its activities were then measured using the oligo(dC)-tailed template assay (Fig. 5B). As we have reported previously, purified Elongin BC complex strongly increased the elongation activity of Elongin A (lanes 2–4). However, the addition of increasing concentrations of Elongin BC had no detectable effect on the activity of Elongin A3 (lanes 5–7).

Therefore, to identify the sequences important for the transcriptional activation by Elongin BC, several Elongin A3 mutants were constructed (Fig. 5A). A3M is a mutant Elongin A3 that contains valine and alanine in place of two glycine residues (Gly-499 and Gly-502) within the ATP-binding motif at its COOH terminus. These amino acid substitutions were actually effective in abolishing the kinase activity of one of the serine/threonine kinases, TIP30 (29). A3-A(532) and A3-A(490) are chimeric proteins in which the NH2-terminal
regions of Elongin A3 have been fused to the COOH-terminal regions of Elongin A. The former includes residues 1–317 of Elongin A3 and residues 532–772 of Elongin A, whereas the latter includes residues 1–276 of Elongin A3 and residues 490–772 of Elongin A. These mutant proteins were then expressed in insect cells, purified, and tested for transcriptional activity and responsiveness to Elongin BC. As shown in Fig. 5C, all of the mutant proteins possessed comparable levels of transcriptional activity to the wild-type Elongin A3 in the absence of Elongin BC. Among the mutants tested, A3M, A3-A-A3, and A3-A (532) were unresponsive to Elongin BC (lanes 1–7). In contrast, A3-A (490) was significantly activated by Elongin BC (lanes 8–10).

The results described above suggest that the potential ATP binding site is unlikely to be required for the transcriptional activity of Elongin A3, although this region may play an in vivo regulatory role not yet revealed by in vitro assays. Moreover, they demonstrate that the COOH-terminal region between residues 490 and 772 of Elongin A, which contains the Elongin BC-binding motif and the sequences outside this region, is responsible for the transcriptional activation by Elongin BC. In a previous study, we demonstrated that residues 520–680 of Elongin A are essential for its transcriptional activity by a systematic structure-function analysis (16). In those experiments, because most of the assays were performed in the presence of Elongins B and C, we could not clarify whether the activity represents that of Elongin A itself or induction by Elongin BC. At the present time, we do not know whether the entire region from residues 490 to 772 or some portion of this region is required for the induction. Further investigation is necessary to determine the most critical sequence substitutions in Elongins A2 and A3 for the loss of responsiveness to Elongin BC.

What is the mechanism by which Elongin A is activated by Elongin BC? Although information concerning the structure of mammalian Elongin A is lacking, Koth et al. (30), using circular dichroism, recently analyzed the structures of the Elongin A and C subunits from yeast *Saccharomyces cerevisiae* in which Elongin A possesses no transcriptional activity and no Elongin B subunit is present. According to them, yeast Elongin A is unfolded in the absence of Elongin C, and there is a large increase in helical content upon formation of the Elongin AC heterodimer. If this is also the case in mammalian Elongins, the activation of Elongin A might be induced by an allosteric mechanism in which the binding of Elongin BC causes the Elongin A elongation activation domain to adopt a more transcriptionally active conformation.

Elongin A3 and Elongin A Do Not Act in a Synergistic Manner—The results of Northern blot analysis and studies from other laboratories suggest that both Elongin A and Elongin A3 are expressed in most mammalian tissues and cell types (10, 28, 31–33). It has also been reported that Elongin A is predominantly localized in the nucleus (25, 34). Thus, to investigate the subcellular localization of Elongin A3 we performed immunostaining assays. COS7 cells were transfected with a construct expressing FLAG-tagged Elongin A3 and stained with anti-FLAG antibody. As shown in Fig. 6A, Elongin A3 was clearly localized to the nucleus and displayed a dot-like distribution pattern. These results suggest that Elongin A3 and Elongin A might coexist in the cells.

Therefore, we next tested the effect of Elongin A3 on the transcriptional activity of Elongin A using an oligo(dC)-tailed template assay (Fig. 6B). In this experiment, we used a limiting concentration of purified RNA polymerase II compared with Elongins A3 and A. In the absence of Elongin, a substantial portion of the −135-nucleotide transcripts persisted for at least 7 min after the addition of UTP and nonradioactive CTP (lanes 1). In the presence of either 4 nM purified Elongin A3 or 8 nM...
purified Elongin A (lanes 2 and 3), nearly all of the ∼135-nucleotide transcripts had been chased into the longer transcripts. Notably, the transcripts synthesized in the presence of Elongin A were substantially longer than those synthesized in the presence of Elongin A3. However, the addition of Elongin A3 to Elongin A resulted in a substantial inhibition of Elongin A activity, and RNA chain elongation progressed at an intermediate rate (lane 4). These results suggest that Elongin A3 and Elongin A act not in a complementary way but rather via a similar mechanism. Although we do not know the mechanism of this action, the two may share the surface of RNA polymerase II.

What might be the roles of members of the Elongin A family in vivo? Because Elongin A is capable of stimulating the rate of elongation through a wide variety of DNA templates tested in vitro, this factor has been considered to be a general elongation factor. However, cDNA microarray analyses using the wild-type and Elongin A-deficient ES cells indicate that the expression of only a small percentage of genes is significantly reduced in the mutant cells. These findings suggest that members of the Elongin A family are not general elongation factors and that each factor regulates the expression of a distinct set of genes. Furthermore, it is also possible that Elongins A2 and A3 work as negative regulators of Elongin A in vivo by binding to Elongin BC and inhibiting its ability to activate the transcriptional activity of Elongin A. Alternatively, the Elongin A family could have some other cellular functions. It was recently reported that the Elongin A-Elongin BC complex is capable of assembling with known components of the ubiquitin ligase, Cul5 and Rbx1 (23). We have also found that both Elongins A2 and A3 are capable of assembling with these proteins as efficiently as Elongin A (data not shown). These findings raise the intriguing possibility that at least one of the functions of the Elongin A family may be to participate in ubiquitin-dependent degradation of components of the RNA polymerase II elongation machinery.

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