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Identification of EloA-BP1, a novel Elongin A binding protein with an exonuclease homology domain

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Abstract

The Elongin complex stimulates the rate of transcription elongation by RNA polymerase II by suppressing the transient pausing of the polymerase at many sites along the DNA template. Elongin is composed of a transcriptionally active A subunit, and two positive regulatory B and C subunits. Although the NH_2-terminal ~120 amino acid region of Elongin A is dispensable for its transcriptional activity in vitro, it shares significant sequence similarity with the NH_2-terminus of other class of transcription factors SII and CRSP70, suggesting that the NH_2-terminus mediates interactions important for the regulation of transcription in vivo. To identify proteins that can bind to these conserved sequences, a human B cell cDNA library was screened using the NH_2-terminus of Elongin A as bait in a yeast two-hybrid system. Here, we report on the cloning and characterization of a novel human exonuclease domain-containing protein, Elongin A-binding protein 1 (EloA-BP1). EloA-BP1 is composed of 1221 amino acids and its mRNA is ubiquitously expressed. Double immunofluorescence labeling in COS7 cells suggested that EloA-BP1 and Elongin A are colocalized to the cell nucleus. By using an in vitro binding assay, we show that EloA-BP1 is capable of binding not only the NH_2-terminal ~120 amino acid region of Elongin A, but also that of SII. Although the purified EloA-BP1 had no detectable effect on the rate of transcription elongation in vitro, it may play some role in the regulation of elongation in vivo.

Keywords: Elongin; SII; Transcription elongation; RNA polymerase II; Exonuclease

The synthesis of messenger RNA in eukaryotes is a complex biochemical process controlled by the concerted action of a diverse collection of transcription factors that regulate the activity of RNA polymerase II during the initiation and elongation stages of transcription [1–5]. Elongin is a member of a class of transcription elongation factors, which all function to increase the overall rate of RNA chain elongation by RNA polymerase II by decreasing the frequency and/or duration of transient pausing of the polymerase at sites along the DNA template [6–8]. The other class of elongation factors is composed solely of members of the SII family. SII reactivates arrested RNA polymerase II by promoting endonucleolytic cleavage of the nascent transcript [9].

Elongin is a heterotrimer composed of A, B, and C subunits of ~770, 118, and 112 amino acids, respectively [7,10,11]. Elongin A is the transcriptionally active component of the Elongin complex, whereas Elongins B and C are positive regulatory subunits. The structure and function of Elongin A have been evolutionarily conserved [7,12–16]. The NH_2-terminus of both the mammalian and *Caenorhabditis elegans* Elongin A subunits shares significant sequence similarity with an ~120 amino acid region of elongation factor SII and the 70 kDa subunit of the coactivator complex CRSP (CRSP70) [17,18]. Despite this sequence similarity, the NH_2-terminus of both Elongin A and SII is dispensable for the transcriptional activity in vitro [12,19,20]. Thus,

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the presence of these conserved sequences in several different classes of transcription factors suggests that their NH_2-termini may mediate interactions important for the regulation of transcription in vivo.

To better understand the function of these shared sequences, we attempted to identify proteins that can associate with the NH_2-terminus of Elongin A using a yeast two-hybrid system. Here we describe the molecular cloning of a novel Elongin A-binding protein (EloA-BP1), which can directly interact with the NH_2-terminus of both Elongin A and SII. EloA-BP1 is composed of 1221 amino acids and contains an exonuclease domain at its COOH-terminus. We also describe the initial characterization of EloA-BP1.

Materials and methods

Yeast two-hybrid screening. cDNA encoding amino acids 1–172 of rat Elongin A was amplified by polymerase chain reaction (PCR) and subcloned in-frame using the *Nde*I and *Bam*HI sites downstream of the GAL4 DNA binding domain in the plasmid pAS2-1 (Clontech). The resultant plasmid, pAS2-1-Elongin A, carrying the *TRP1* marker was used to transform yeast strain Y190, which contains two reporter genes, *HIS3* and *lacZ*, under the control of a promoter containing the GAL1 binding site, and the transformant was named Y190/pAS2-1-Elongin A. A pACT human B cell cDNA library was transformed into Y190/pAS2-1-Elongin A with *LEU2* marker selection for the library plasmid and the resultant transformants were screened for two-hybrid interactions as previously described [21]. Seven colonies out of 1.4×10^7 transformants showed the His+ *lac*Z+ phenotype. The identity of the cDNAs recovered from these positive yeast strains was determined by sequencing the insert DNA.

Plasmid construction. FLAG-tagged Elongin A was amplified by PCR from pSVL-Elongin A [22] and subcloned into the *Nhe*I and *Bam*HI sites of pcDNA3.1(+) (Invitrogen). The baculovirus vector encoding FLAG-tagged Elongin A was described previously [15]. The cDNA encoding full-length EloA-BP1 (Gene name: KIAA1138, GenBank Accession No.: AB032964) was obtained from Kazusa DNA Research Institute (Kisarazu, Japan). HA-tagged EloA-BP1 was amplified by PCR from KIAA1138 and subcloned into *Nhe*I and *Eco*RI sites of pCI-neo (Promega) or *Xba*I and *Eco*RI sites of pBacPAK-His2 (Clontech). Subsequently, since the expression level of the full-length EloA-BP1 protein was very low for unknown reasons, constructs expressing a NH_2-terminal deletion mutant (ΔN-EloA-BP1) lacking amino acids 4–489 of EloA-BP1 were generated by oligonucleotide-directed mutagenesis of pCI-EloA-BP1 or pBacPAK-His2-EloA-BP1 using the QuickChange site-directed mutagenesis kit (Stratagene). For expressing glutathione S-transferase (GST) fusion proteins, cDNA encoding amino acids 1–120 of Elongin A, amino acids 1–115 of SII, or amino acids 499–580 of EloA-BP1 was amplified by PCR from pSVL-Elongin A, pET23d-SII (a gift from Dr. Z.F. Burton), or KIAA1138, respectively, and subcloned into the *Bam*HI and *Sal*I sites of pGEX-4T1 (Amersham).

DNA sequencing and Northern blot analysis. DNA sequencing was performed using an automated sequencer (ABI Prism 310, Applied Biosystems). Human multiple tissue Northern blot (Clontech), containing 2 μg of poly(A)$^+$ RNA per sample, was hybridized using ExpressHyb solution (Clontech) under stringent conditions as recommended by the manufacturer. The EloA-BP1 probe contained sequences encoding amino acids 1–1221, and the blot was stripped and reprobed using β-actin cDNA.

Expression of recombinant proteins in Escherichia coli and in vitro binding assay. GST and GST fusion proteins were expressed in *E. coli* DH5α and purified by glutathione–Sepharose affinity chromatography (Amersham) according to the manufacturer's instructions. pcDNA3.1-Elongin A and pCI-ΔN-EloA-BP1 were used for in vitro transcription and translation (TNT coupled reticulocyte lysate systems, Promega). Immobilized GST fusion proteins (~15 μg) were mixed with 15 μl of in vitro translated [^{35}S]methionine-labeled lysate and incubated for 1 h at 4 °C. The beads were then washed five times with buffer containing 20 mM Hepes–NaOH (pH 7.9), 500 mM NaCl, 2.5 mM $MgCl_2$, 50 μM $ZnCl_2$, 0.1 mM EDTA, 0.05% Nonidet P-40, and 0.5 mM DTT. Bound protein was separated on an 8.0% SDS–polyacrylamide gel and detected by fluorography.

Expression of recombinant proteins in insect cells. Recombinant baculoviruses were generated using the BacPAK baculovirus expression system (Clontech). Sf9 cells were cultured in Grace's insect medium (GIBCO) supplemented with 10% fetal bovine serum at 27 °C and infected with the indicated recombinant baculoviruses. Seventy hours after infection, the cells were collected and lysed as described previously [15,16]. The cell lysates were then centrifuged at 10,000*g* for 20 min and the supernatants were used for immunoprecipitation and protein purification. ΔN-EloA-BP1 with 6-histidine and HA tags was purified consecutively using Ni^{2+}–agarose (Invitrogen) and anti-HA conjugated agarose beads (Roche). Rat Elongin A with 10-histidine and FLAG tags was purified as described [15]. Aliquots of the eluates were used for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and in vitro transcription elongation assay.

Immunoprecipitation and Western blotting. Baculovirus-infected cell lysates were incubated with an appropriate antibody for 1 h at 4 °C and then with protein A–Sepharose CL-4B for 1 h at 4 °C. The beads were washed four times with buffer containing 40 mM Hepes–NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, and 10% glycerol. Immunoprecipitated proteins were subjected to SDS–PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and visualized by Western blotting using a chemiluminescence reagent (NEN).

Immunofluorescence staining. COS7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and were transiently transfected with pcDNA3.1-FLAG-Elongin A and pCI-HA-ΔN-EloA-BP1 using FuGENE 6 (Roche) according to the manufacturer's protocol. The cells grown in a chamber slide were fixed by immersion in 100% ethanol for 30 min and then rinsed with 70% ethanol, 50% ethanol, and finally phosphate-buffered saline (PBS). After blocking in PBS containing 2% bovine serum albumin, 0.2% Tween 20, and 6.7% glycerol at 4 °C overnight, the cells were incubated for 1 h at 4 °C with rabbit polyclonal anti-FLAG antibody (1:1000) and mouse monoclonal anti-HA antibody. Reacting antibodies were stained with fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG_{2b} (Santa Cruz). Fluorescence microscopy of fixed cells was performed using an Olympus BX-50 confocal laser scanning microscope.

Oligo(dC)-tailed template assay of transcription elongation by RNA polymerase II. RNA polymerase II was purified as described from rat liver nuclear extracts [23]. Pulse-chase assays were carried out essentially as reported previously [7]. RNA polymerase II (0.01 U) and 100 ng pCpGR220S/P/X were incubated at 28 °C in the presence of 20 mM Hepes–NaOH (pH7.9), 20 mM Tris–HCl (pH7.9), 2% (w/v) polyvinyl alcohol, 0.5 mg/ml of bovine serum albumin, 60 mM KCl, 50 μM $ZnSO_4$, 7 mM $MgCl_2$, 0.2 mM DTT, 3% (v/v) glycerol, 3 U of recombinant ribonuclease inhibitor, 50 μM ATP, 50 μM GTP, 2 μM CTP, and 10 μCi [α-^{32}P]CTP. After 20 min labeling, 100 μM non-radioactive CTP, 2 μM UTP, and specific amounts of Elongin A and EloA-BP1 preparations were added, and the reactions were incubated for 7 min. Transcripts were analyzed by electrophoresis through 6% polyacrylamide–7.0 M urea gels.

Results and discussion

Identification of a novel Elongin A-binding protein with an exonuclease homology domain

To identify proteins that interact with the NH_2-terminus of Elongin A, a yeast two-hybrid system was employed. The cDNA encoding amino acids 1–172 of Elongin A was subcloned into the yeast expression vector pAS2-1, to yield pAS2-1-Elongin A. A pACT human B cell cDNA library was transformed into the Y190 strain carrying pAS2-1-Elongin A. Interaction between the bait protein and a protein encoded by the cDNA library was identified by growth on medium lacking histidine and the expression of β-galactosidase in a X-gal filter assay. Approximately 1.4×10^7 independent transformants of the cDNA library were screened, and seven colonies that showed both growth on medium lacking histidine and production of β-galactosidase in a filter assay were identified. The DNA sequencing of the cDNAs isolated from positive colonies demonstrated that these seven clones contained identical insert DNA encoding amino acids 499–580 of the uncharacterized human KIAA1138 protein [24]. Thereafter, we designated KIAA1138 protein as EloA-BP1. The full-length EloA-BP1 is composed of 1221 amino acids with a calculated molecular mass of 131,524 Da (Fig. 1A). Although EloA-BP1 contains no structural motifs

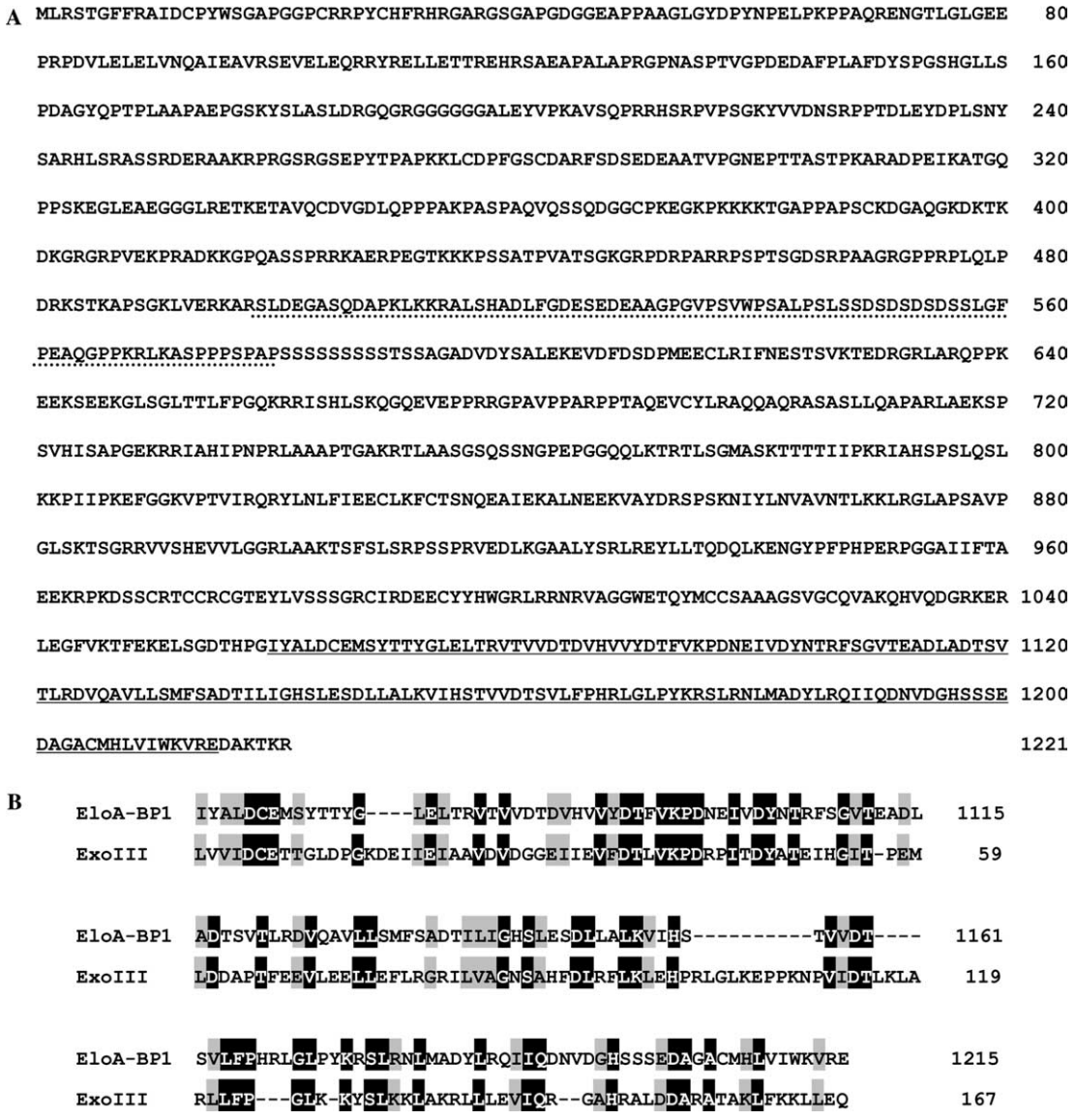

Fig. 1. The primary structure of human EloA-BP1. (A) Predicted amino acid sequence of EloA-BP1. The region required for binding to Elongin A is indicated by a dashed underline. The exonuclease homology domain present at the COOH-terminus of EloA-BP1 is underlined. (B) Sequence similarity between EloA-BP1 and the exonuclease domain (Exo III). Identical amino acids are shown in white letters on a black background and chemically similar amino acids are shown in black letters on a gray background. Numbers indicate amino acid positions in each protein.

characteristic of transcription factors, such as leucine zipper, zinc finger, or helix-turn-helix domains, its COOH-terminus shows striking sequence similarity with the exonuclease domain found in various factors such as the α and ε subunits of DNA polymerase III, and ribonuclease T [25]. An alignment of the COOH-terminus of EloA-BP1 (residues 1060–1215) and exonuclease domain (Exo III) indicates that 35% of the amino acids are identical and 50% are similar over a 149 amino acid overlap (Fig. 1B).

EloA-BP1 directly associates with the NH_2-terminal portions of Elongin A and SII

A positive interaction in the two-hybrid assay may reflect a direct association between bait and prey or an indirect association mediated by an endogenous bridging protein. To confirm that the interaction detected in the present screening is due to a direct association between Elongin A and EloA-BP1, an in vitro binding assay was performed. Amino acid residues 499–580 of EloA-BP1, which correspond to the central portion of this protein and were contained in all of the isolated cDNA clones were fused to GST, expressed in *E. coli*, and immobilized on glutathione–Sepharose resin. The GST-EloA-BP1(499–580) resin was incubated with in vitro translated ^{35}S-labeled Elongin A. After extensive washing, the bound protein was eluted, fractionated by SDS–PAGE, and visualized by autoradiography. As shown in Fig. 2A, Elongin A was retained by the GST-EloA-BP1(499–580) affinity resin, while no detectable Elongin A was retained by the resin containing GST alone, suggesting that this association is mediated by direct interaction between the central portion of EloA-BP1 and Elongin A.

We next sought to test whether full-length EloA-BP1 is also capable of interacting with Elongin A. However, full-length EloA-BP1 was not efficiently expressed in either *E. coli* or Sf9 cells, so we generated a baculovirus expressing an NH_2-terminal deletion mutant that lacks amino acids 4–489 of EloA-BP1 (ΔN-EloA-BP1). HA-tagged ΔN-EloA-BP1 was coexpressed with FLAG-tagged Elongin A in Sf9 insect cells and its ability to bind Elongin A was assessed in a coimmunoprecipitation experiment. As shown in Fig. 2B, Elongin A was coprecipitated with ΔN-EloA-BP1 by anti-HA antibody, suggesting that the longer form of EloA-BP1 containing 735 amino acids is also capable of forming a stable complex with Elongin A in cells.

The NH_2-terminus of Elongin A used as the bait in our screening contained sequences homologous to the NH_2-termini of transcription factors SII and CRSP70 [17,18]. The NH_2-terminal ~120 amino acids of Elongin A and SII are 29% identical and 53% similar over a 108 amino acid overlap [7]. Booth et al. [26] recently determined the structure of SII from yeast *Saccharomyces*

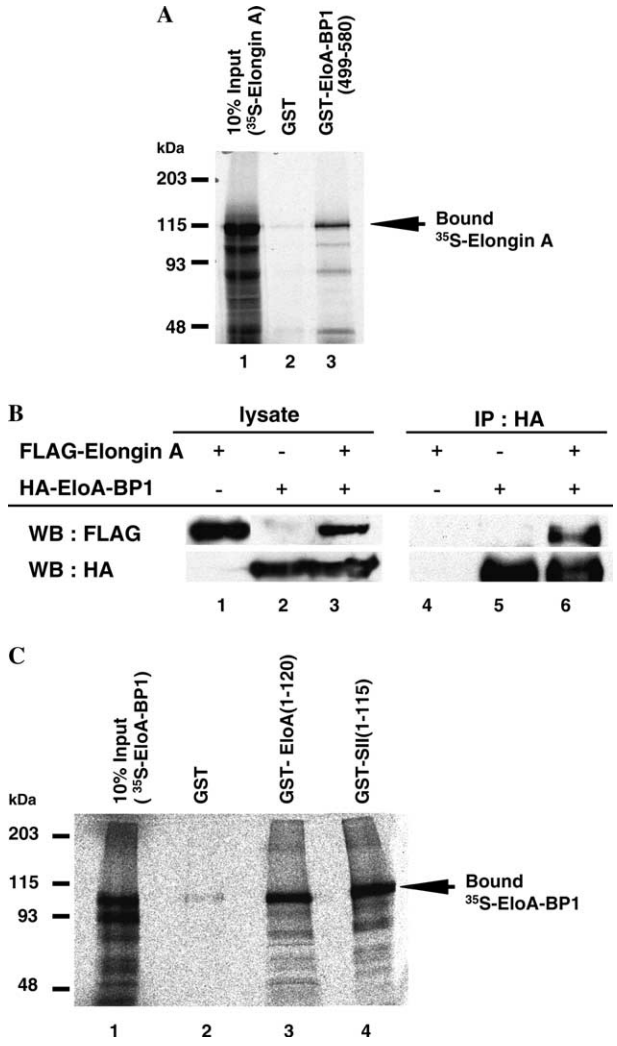

Fig. 2. Interaction of Elongin A and EloA-BP1. (A) [^{35}S]methionine-labeled full-length Elongin A was incubated with GST (lane 2) or GST-EloA-BP1(499–580) (lane 3). After extensive washing the bound proteins were eluted, resolved by 8% SDS–PAGE and visualized by autoradiography. Ten percent of the total input of [^{35}S]methionine-labeled full-length Elongin A is shown in lane 1. (B) Lysates from Sf9 cells expressing the indicated baculoviruses were immunoprecipitated with anti-HA antibody. Immunoprecipitated proteins were detected by Western blotting using the indicated antibodies. (C) [^{35}S]methionine-labeled ΔN-EloA-BP1 was incubated with GST (lane 2), GST–Elongin A(1–120) (lane 3), or GST-SII(1–115) (lane 4). After extensive washing the bound proteins were eluted, resolved by 8% SDS–PAGE, and visualized by autoradiography. Ten percent of the total input of [^{35}S]methionine-labeled ΔN-EloA-BP1 is shown in lane 1.

cerevisiae using NMR spectroscopy and modeled the NH_2-terminal regions of Elongin A and CRSP70 using the structure of SII as a template. According to them, the NH_2-terminus of all three proteins formed a four-helix bundle, and the hydrophobic core residues of helices and several surface residues were well conserved among SII, Elongin A, and CRSP70. Therefore, to investigate the possibility that these conserved sequences are responsible for binding to EloA-BP1, amino acid residues 1–120 of Elongin A or residues 1–105 of SII,

were fused to GST, expressed in *E. coli*, and immobilized on glutathione–Sepharose resin. These resins were then incubated with in vitro translated ^{35}S-labeled ΔN-EloA-BP1. After extensive washing, the bound protein was eluted, fractionated by SDS–PAGE, and visualized by autoradiography. As shown in Fig. 2C, ΔN-EloA-BP1 was retained by the GST-Elongin A(1–120) and GST-SII(1–115) affinity resins, while no detectable ΔN-EloA-BP1 was retained by the resin containing GST alone. Thus, as predicted from the sequence homology, the NH_2-terminal ~120 amino acid sequence of both Elongin A and SII is capable of binding to EloA-BP1.

EloA-BP1 is ubiquitously expressed

To examine the tissue distribution of EloA-BP1, Northern blots containing poly(A)$^+$ RNA from various human tissues were hybridized with an EloA-BP1-specific probe. As shown in Fig. 3, the EloA-BP1 probe hybridized to a single band of ~4.4 kb. Although the intensity of the signals varied among tissues, this mRNA species was detected in all of the tissues examined. These results suggest that EloA-BP1 is ubiquitously expressed in human tissues and may play fundamental roles in cells.

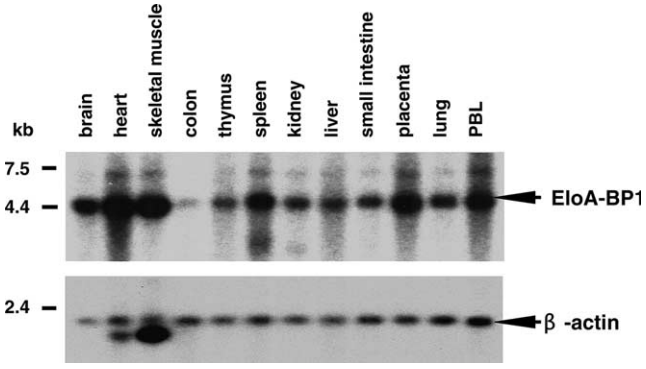

Fig. 3. Expression of EloA-BP1 in human tissue. Multiple tissue Northern blot (Clontech) containing poly(A)$^+$ RNA of the indicated human tissues was hybridized with an EloA-BP1 (upper) or β-actin cDNA probe (lower). The size of the RNA standards is indicated on the left. *PBL*, peripheral blood leukocyte.

EloA-BP1 and Elongin A colocalize in the cell nucleus

The results of Northern blot analysis and studies from other laboratories suggest that Elongin A is expressed in most mammalian tissues and cell types [14,15,27–29]. It has also been reported that Elongin A is predominantly localized to the cell nucleus [30]. Thus, we next examined whether EloA-BP1 and Elongin A associate in cells by coexpressing FLAG-tagged Elongin A and HA-tagged EloA-BP1 in COS7 cells. Transfected COS7 cells were doubly stained with anti-FLAG (green) and anti-HA (red). As shown in Fig. 4, Elongin A was expressed diffusely in the nucleus. Anti-HA (EloA-BP1) staining showed nuclear staining that was almost indistinguishable from that of anti-FLAG (Elongin A). Merging the two images produced yellow, suggesting that Elongin A and EloA-BP1 colocalize in the cell nucleus.

EloA-BP1 has no detectable effect on transcription elongation in vitro

Therefore, we next tested whether EloA-BP1 has any effect on the rate of transcription elongation in vitro. To do this, we first expressed recombinant ΔN-EloA-BP1 with 6-histidine and HA tags at the NH_2-terminus in insect cells and purified it to near homogeneity by sequential Ni^{2+}–agarose and anti-HA–agarose affinity chromatography. Recombinant ΔN-EloA-BP1 had an apparent molecular mass of 100 kDa (Fig. 5A).

Then, the purified ΔN-EloA-BP1 at increasing concentrations was preincubated at 4 °C for 30 min in the

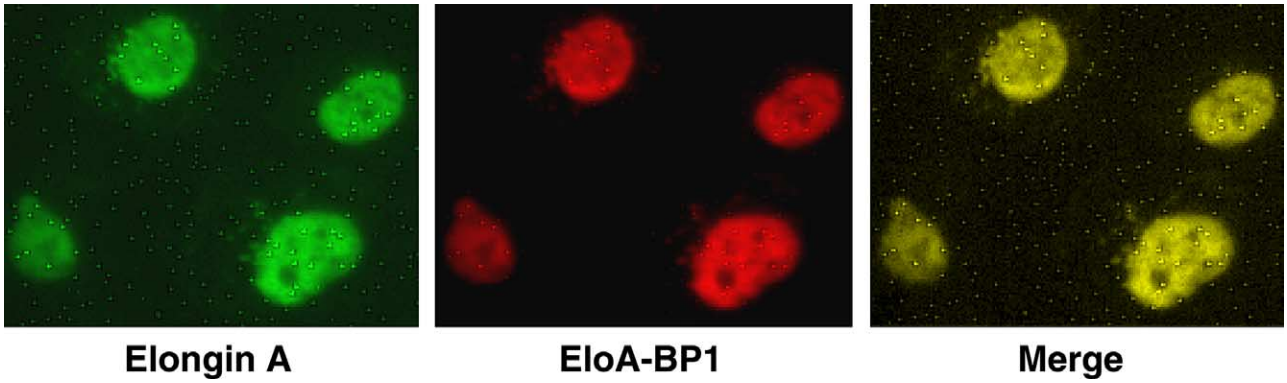

Fig. 4. Subcellular localization of EloA-BP1 and Elongin A. COS7 cells were transfected with vectors expressing FLAG-tagged Elongin A and HA-tagged EloA-BP1. After 12 h, the cells were stained for Elongin A using anti-FLAG antibody (green) (left panel) and EloA-BP1 using anti-HA antibody (red) (middle panel). A merged image is shown (right panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

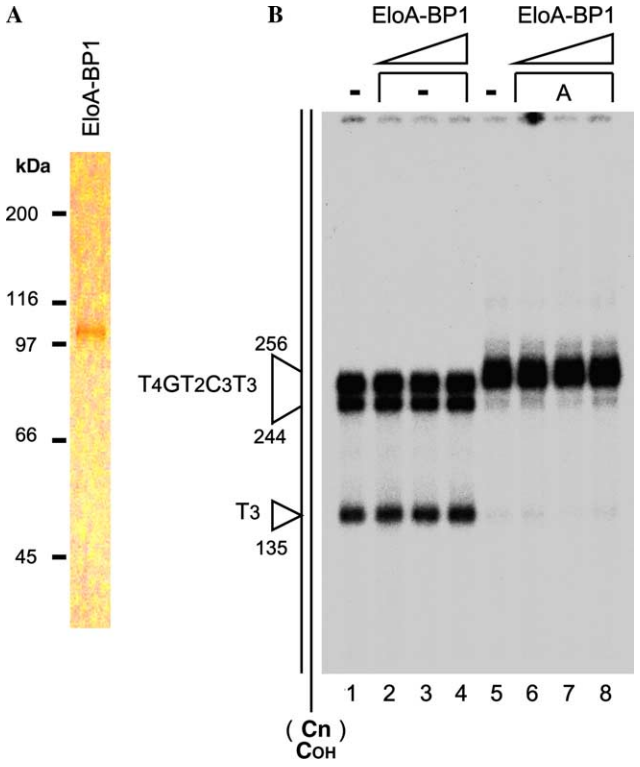

Fig. 5. Effect of EloA-BP1 on the transcriptional elongation activity of Elongin A. (A) ΔN-EloA-BP1 expressed in and purified from insect cells as described under "Materials and methods" was subjected to 8% SDS–PAGE. The protein was visualized by silver staining. (B) The oligo(dC)-tailed template assays were performed in the absence (lanes 1–4) or presence of 3 nM Elongin A (lanes 5–8) purified from insect cells. ΔN-EloA-BP1was present in reaction mixtures at 1.5 nM (lanes 2 and 6), 3 nM (lanes 3 and 7), or 6 nM (lanes 4 and 8).

absence or presence of purified Elongin A and the activity was measured using the oligo(dC)-tailed template assay (Fig. 5B). In this experiment, transcription was initiated by the addition of RNA polymerase II to reaction mixtures containing ATP, GTP, and [α-^{32}P]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 244–256 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 non-radioactive CTP, in the absence or presence of purified Elongin A and ΔN-EloA-BP1. In the absence of Elongin A, a substantial portion of the ~135-nucleotide transcripts persisted for at least 7 min after the addition of UTP, and none of them had been chased into longer transcripts in the presence of increasing concentrations of ΔN-EloA-BP1 (lanes 1–4). In the presence of Elongin A, RNA chain elongation progressed more rapidly, with nearly all of the transcripts chased into ~250-nucleotide transcripts within 7 min after the addition of UTP, while the presence of increasing concentrations of ΔN-EloA-BP1 had no detectable effect on the kinetics of the synthesis (lanes 5–8). These results suggest first that ΔN-EloA-BP1 had no direct effect on the rate of transcription elongation by purified RNA polymerase II in vitro, and second that ΔN-EloA-BP1 had neither stimulatory nor inhibitory effects on the transcriptional activity of Elongin A in vitro. Although at present we do not know the precise reason why ΔN-EloA-BP1 had no detectable effect on transcription elongation, it might be because the NH_2-terminus of EloA-BP1, which was missing in ΔN-EloA-BP1, is essential for its activity or some additional factor is required for the effect of EloA-BP1 on transcription elongation.

What might be the role of EloA-BP1 in vivo? Pan et al. [31] have shown that the NH_2-termini of Elongin A and SII are capable of binding to an RNA polymerase II holoenzyme complex containing multiple general initiation factors. Thus, it is possible that EloA-BP1 is an as yet to be identified component of the RNA polymerase II holoenzyme complex that is responsible for direct interaction with the NH_2-terminus of both Elongin A and SII. Alternatively, EloA-BP1 is not a component of the holoenzyme complex, but functions as a bridging molecule that mediates the interaction between the NH_2-terminus of Elongin A or SII and the holoenzyme complex. Furthermore, the striking sequence similarity of the COOH-terminal region of EloA-BP1 with an exonuclease domain raises the intriguing possibility that EloA-BP1 has a proofreading function during transcription elongation by RNA polymerase II. In this scenario, EloA-BP1 might promote exonucleolytic cleavage of the 3′-OH-terminus of the nascent RNA transcript to remove misincorporated nucleotides and help to maintain the fidelity of the transcriptional products. Experiments to investigate these possibilities are underway.

Acknowledgments

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