Mammalian Elongin A Is Not Essential for Cell Viability but Is Required for Proper Cell Cycle Progression with Limited Alteration of Gene Expression

Katsuhisa Yamazaki, Teiji Aso, Yoshinori Ohnishi, Mizuki Ohno, Kenji Tamura, Taro Shuin, Shigetaka Kitajima, and Yusaku Nakabepu

From the Medical Institute of Bioregulation, Kyushu University, and CREST, Japan Science and Technology Corporation, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, the Department of Biochemical Genetics, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, and the Departments of Chemistry and Urology, Faculty of Medicine, Kochi Medical School, Kochasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

Elongin A is a transcription elongation factor that increases the overall rate of mRNA chain elongation by RNA polymerase II. To investigate the function of Elongin A in vivo, the two alleles of the Elongin A gene have been disrupted by homologous recombination in murine embryonic stem (ES) cells. The Elongin A-deficient ES cells are viable, but show a slow growth phenotype because they undergo a delayed mitosis. The cDNA microarray and RNase protection assay using the wild-type and Elongin A-deficient ES cells indicate that the expression of only a small subset of genes is affected in the mutant cells. Taken together, our results suggest that Elongin A regulates transcription of a subset but not all of genes and reveal a linkage between Elongin A function and cell cycle progression.

Eukaryotic messenger RNA synthesis by RNA polymerase II (pol II) is regulated by the concerted action of a set of general transcription factors that control the activity of pol II during the initiation and elongation stages of transcription. At least six general transcription initiation factors (TFIID, TFIIF, TFIIE, TFIIF, and TFIHII) have been identified in eukaryotic cells and found to promote the selective binding of pol II to promoters and to support a basal level of transcription (1). In addition to the general initiation factors, at least 17 elongation factors have been defined biochemically and found to promote efficient elongation of transcripts by pol II (2–5). These elongation factors fall into two broad functional classes based on their abilities to either reactivate arrested pol II or suppress transient pausing of pol II. The first class is solely composed of SII (6). The rest of the elongation factors, including TFIIF (7), Elongin A (8), Elongin A2 (9), Elongin A3 (10), and Cockayne syndrome B (CSB) protein (11), all act to increase the overall rate of mRNA chain elongation by decreasing the frequency and/or duration of transient pausing of pol II at sites along the DNA template.

Elongin was initially identified as a heterotrimer composed of A, B and C subunits of ~770, 118, and 112 amino acids, respectively (8, 12–14). Elongin A is the transcriptionally active subunit of the Elongin complex, whereas Elongins B and C are positive regulatory subunits. Biochemical studies have shown that Elongins B and C form a stable Elongin BC complex that binds to Elongin A and strongly induces its transcriptional activity (8, 15). Elongin C functions as the inducing ligand and activates transcription through interaction with a short conserved motif (consensus sequence (T,S)LXXXXX(V,L,I)) in the elongation activation domain of Elongin A (15). Elongin B, a member of the ubiquitin homology protein family, appears to play a chaperone-like role in the assembly of the Elongin complex by binding to Elongin C and facilitating its interaction with Elongin A (8, 14). Notably, Elongins B and C are also found as integral components of a multisubunit complex containing the von Hippel-Lindau (VHL) tumor suppressor protein (16, 17). Elongin A and the VHL protein share the Elongin BC binding site motif; and consistent with the assumption of a role for Elongin BC in tumor suppression, more than 70% of VHL mutations found in VHL families and sporadic clear cell renal carcinomas are associated with a mutation or deletion at this site (16, 18).

To gain more insight into the role of Elongin A in vivo, we have inactivated both alleles of the Elongin A gene in mouse ES cells. We found that Elongin A is not essential for the viability of ES cells. Though, surprisingly, such Elongin A-deficient ES cells display several abnormalities, in cell size, cell growth, and cell cycle distribution, which are similar to those of cells with a delayed onset of mitosis. Furthermore, the absence of Elongin A protein affected the expression of only a subset of class II genes, indicating that mammalian Elongin A seemed to be required for pol II transcription of a subset but not all of genes.

Experimental Procedures

Generation of the Elongin A-deficient ES Cell Clones—A 15-kb genomic fragment spanning the sequence encoding exons 2–11 from the 3′-UTR of murine Elongin A was isolated from a 129/Sv mouse genomic library, using a mouse Elongin A cDNA probe (19). Genomic fragments of 4.5 and 1.4 kb flanking exons 8–10 of Elongin A were used to
generate a targeting construct in which exons 8–10 were replaced by a pol II-neo-poly(A) cassette (20). To increase the frequency of gene targeting, a pair of herpes simplex virus (HSV-1 and HSV-2 thymidine kinase (TK) cassettes (TK1 and TK2, both under control of the MC1 promoter) was placed flanking the Elongin A genomic sequence in the targeting vector (21, 22). CCE ES cells were electroporated with the Sall-linearized targeting vector as described previously (23, 24), and heterozygous Elongin A-deficient clones were selected in the presence of 0.3 mg/ml G418 (Sigma) and 5 μg ganciclovir (Japan Syntechs). Among 200 ES cell colonies, three correctly targeted clones were identified by Southern blot analysis, using 5′ and 3′ external probes located outside the homologous regions of the targeting vector. Subsequently, homozygous Elongin A-deficient clones were obtained by selection in higher concentrations of G418 (from 1.5 to 2.0 mg/ml).

**Southern Blot Analysis**—Genomic DNA was isolated from ES cells, digested with either XbaI or BamHI, separated on a 0.65% agarose gel, and transferred to nylon membranes (Amersham Biosciences) by capillary blotting. The membrane was hybridized with random-primed [32P]-labeled external probe (Fig. 1A) by using standard methods.

**RT-PCR Analysis**—Total RNA was prepared from cultured cells using ISOGEN (Nippon Gene). First-strand cDNA was synthesized using a first-strand cDNA synthesis kit (Amersham Biosciences) as described in the manufacturer’s instructions. Subsequently, PCR was performed with primers A1 (5′-GCGTGGAGAGTTGCTCTTG-3′) and A2 (5′-TTATCGCCGGGAGAATCTGTTCTTGAATG-3′) to confirm the deletion of Elongin A. RT-PCR analysis was performed with primers mGA1 (5′-CTGCCATTGGCAGTTGGC-3′) and mGA2 (5′-TGTTATCCAGAGATTAGGGA-3′).

**Cell Proliferation Assay**—In vitro cell growth was assessed by plating 1 × 10⁶ cells per well in six-well tissue culture dishes in triplicate. Cells were harvested at 24-h intervals by trypsinization and counted using a hemocytometer.

**Fluorescence-activated Cell Sorting (FACS) Analysis**—FACS analysis was performed as described previously (25, 26). Briefly, ES cells were harvested and fixed with 70% ethanol. After being washed with ice-cold phosphate-buffered saline, fixed cells were stained for cellular DNA with propidium iodide (Sigma). The cell suspension was passed through a nylon mesh membrane and DNA content and cell numbers were analyzed with a FACS Calibur (Becton Dickinson). For each sample, 1 × 10⁶ cells were analyzed, and the results were processed through Cell Quest software (BD Biosciences).

**Cytogenetic Analysis**—Chromosome slides were prepared according to the standard cytogenetic method. Briefly, cultured cells were treated with 1.5 μg/ml TN-16 (3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione, Wako) for 2 h to collect metaphase cells. Cells were harvested using 0.25% trypsin-EDTA and centrifuged at 1000 rpm for 5 min. Cells were treated with hypotonic solution (75 mM KCl) for 20 min at room temperature and fixed with Carnoy’s fixative (methanol:acetic acid = 3:1). Cell suspensions were dropped onto slide glass, then air-dried. Chromosome slides were stained with 3% Giemsa in phosphate-buffered saline for 10–20 min. Chromosome numbers were evaluated using a Zeiss Axioshot microscope under a ×100 objective.

**Microarray Analysis**—Poly(A) RNA was isolated according to the manufacturer’s protocol using a MACS mRNA isolation kit (Miltenyi Biotec). cDNA generation, hybridization, and data collection were performed by Incyte Genomics. In brief, alterations in gene expression were evaluated by reverse transcription of poly(A)+ RNA in the presence of Cy3 or Cy5 fluorescent labeling dyes followed by hybridization to a mouse GEM 2.1 microarray chip. Each chip carries a total of 9509 cDNAs. The data were analyzed using GEOM Tools 2.5 software (Incyte Pharmaceuticals, Inc.).

**RNase Protection Assay**—The RNase protection assay was performed using the mouse cell cycle regulator multiprobe template sets, mCYC-1, mCYC-2, and mCC-1 (BD Biosciences). [32P]UTP-labeled antisense RNA probe was synthesized using T7 RNA polymerase and was purified by phenol-chloroform extractions and ethanol precipitation. Purified probe was mixed with 10 μg of total RNA from ES cells in hybridization buffer and incubated for 16 h at 56°C. Free probe and single-stranded RNA molecules were digested with a mixture of RNase A and T1. The RNase-protected molecules were purified and resolved on a denaturing polycrylamide gel and dried. Autoradiographic signal was scanned on a BAS2000 image analyzer (Fuji), and the signal intensity of each band was quantified using MacBas software (Fuji) and normalized to the corresponding GAPDH levels.

**RESULTS**

To understand the physiological role of Elongin A in vivo, we disrupted the Elongin A gene in mouse ES cells. We first isolated and analyzed the structure of the mouse Elongin A gene (19). A gene-targeting vector was then constructed by replacing exons 8–10 of the Elongin A gene, which encode the domain essential for its transcriptional elongation activity (15), with a pol II-neo-poly(A) cassette (Fig. 1A). Three independent heterozygous Elongin A-deficient ES cell lines were obtained by homologous recombination, and subsequently, homozygous Elongin A-deficient ES cell lines were generated by culturing these heterozygous ES cells in higher concentrations of G418. Genotyping of the isolated clones was determined by Southern blot analysis with flanking 5′- and 3′-UTR probes (Fig. 1B) and confirmed by RT-PCR (Fig. 1C).

To investigate the phenotype of the Elongin A-deficient ES cells, we first compared growth rates of Elongin A-deficient and wild-type ES cells in vitro. As shown in Fig. 2A, homozygous Elongin A-deficient ES cells grew about three times as slowly as wild type ES cells, whereas heterozygous Elongin A-deficient ES cells grew at rates indistinguishable from those of wild-type cells. Moreover, microscopic analysis revealed that the cellular and nuclear sizes of homozygous Elongin A-deficient ES cells were significantly larger than those of wild type and heterozygous deficient ES cells (Fig. 2B and data not shown).

To clarify the cause of the growth defects of the homozygous Elongin A-deficient ES cells, we first used FACS analysis to determine cell cycle profiles. In contrast to wild type and heterozygous Elongin A-deficient ES cells, significantly more G2/M phase cells (or cells with a DNA content of 4N) than G1 phase cells were present in the homozygous Elongin A-deficient population. We also observed that homozygous Elongin A-deficient ES cells, but none of the other cells, included a substantial fraction with a DNA content of 8N (Fig. 3A). The increase of cells with a DNA content of 4N and the appearance of cells with that of 8N in the homozygous Elongin A-deficient population were confirmed by cytogenetic analysis (Fig. 3, B and C). These results imply that the G2/M phase is prolonged in homozygous Elongin A-deficient ES cells and that homozygous Elongin A-deficient ES cells go through DNA rereplication, even after completion of one cycle or DNA replication.

Together, the observed abnormalities in homozygous Elongin A-deficient ES cells, such as increased cell size, more G2/M phase cells, and the presence of products of DNA rereplication, correspond to those of cells with delayed mitosis, which can be caused either by inhibition of G2/M transition or by stimulation of the initiation of DNA replication.

We next intended to determine the mechanism responsible for the observed delayed mitosis. Thus, a cDNA microarray analysis was applied to compare populations and levels of mRNA in ES cells carrying or lacking functional Elongin A. Wild-type or homozygous Elongin A-deficient ES cells were grown to log phase, then poly(A)+ RNA was isolated and used for cDNA synthesis and subsequently hybridized to a microarray chip. Under our experimental conditions, mRNAs whose levels were decreased or increased by 2-fold or greater were considered as differentially expressed transcripts. The expression of the vast majority of genes was unaffected by disruption of the Elongin A gene. However, 241 (2.5%) of the genes assayed had a significantly down-regulated expression in Elongin A-deficient ES cells. Interestingly, a roughly equivalent number of genes was up-regulated by Elongin A inactivation (Fig. 4A and see Supplemental Tables). However, we presently do not know whether this is a consequence of secondary effects due to loss of Elongin A function as a transcription elongation
factor or primary effects caused by loss of Elongin A function, which repress the expression of a subset of genes.

To verify the results of the microarray analysis and also to identify genes responsible for the delayed mitosis phenotype, an RNase protection assay was carried out for quantitative estimation of the expression of cell cycle-related genes in ES cells (Fig. 4B and data not shown). Consistent with the microarray analysis, cyclin E mRNA, but not CDK2, cyclin D2, and cyclin D3 mRNAs, were down-regulated by inactivation of Elongin A. However, the expression of the other tested cell cycle-related genes, including the mRNAs of key regulators of G2/M transition, such as cyclin A2, cyclin B1, cyclin B2, and cdc2, was either not, or was only weakly, altered in Elongin A-deficient ES cells.

DISCUSSION

Extensive study in vitro has demonstrated a requirement for transcription elongation factors in transcriptional regulation; however, the functions of these factors in vivo have not been properly evaluated. Here we report that mammalian cells in which a gene encoding one of the transcription elongation factors, Elongin A, was disrupted by homologous recombination had (i) a delayed mitosis phenotype and (ii) no general, but rather specific, defects in RNA polymerase II-directed transcription.

Although we have not been able to specify how a lack of Elongin A leads to delayed mitosis, we can assume several possible mechanisms. One possibility is that Elongin A is required for the transcription of specific genes involved in G2/M transition, and a reduction in those transcripts caused by Elongin A inactivation results in a delayed onset of mitosis. In this scenario, Elongin A could be a target for gene-specific transcriptional activators and recruited to the promoter regions of specific genes. Indeed, both a gene-selective action and delayed mitosis phenotype following inactivation of general transcription factors have been reported (27–30). TATA-binding protein (TBP) is critical for the transcription of cdc25B (cdc2 phosphatase), and the reduction of cdc25B expression by heterozygous deletion of TBP causes the accumulation of hyperphosphorylated, inactive cdc2, which in turn leads to delayed mitosis (27). Furthermore, human TBP-associated factor 150 (TAF150) or yeast TAF90 is also specifically important for the transcription of cyclin B1 and cyclin A, or SPC98 (a component of the spindle
ES cells. Results from three independent experiments are shown in a graph with error bars representing standard deviations. A, homozygous Elongin A-deficient (−/−) ES cells are larger than wild-type (+/+), heterozygous Elongin A-deficient (+/−) ES cells, and homozygous Elongin A-deficient ES cells. Results from three independent experiments are shown in a graph with error bars representing standard deviations. B, homozygous Elongin A-deficient (−/−) ES cells are larger than wild-type (+/+), heterozygous Elongin A-deficient (+/−), and homozygous Elongin A-deficient ES cells. Cells were stained with Hoechst to visualize nuclei. Magnification, ×320.

**Fig. 3.** Cell cycle profiles of homozygous Elongin A-deficient ES cells are altered. **A,** FACS analysis of wild-type (+/+), heterozygous Elongin A-deficient (+/−), and homozygous Elongin A-deficient (−/−) ES cell clones. DNA content and cell numbers are plotted on the horizontal and vertical axes respectively. The positions of cells with a DNA content of 2N (G1 phase), 4N (G2/M phase), and 8N are indicated. FACS analysis was performed three times with similar results. **B,** quantitative analysis of the number of chromosomes in wild-type (+/+), heterozygous Elongin A-deficient (+/−), and homozygous Elongin A-deficient (−/−) ES cell clones. Two-hundred cells were evaluated for each clone, and data are expressed as the number of cells that contained the indicated number of chromosomes. N on the horizontal axis stands for 20 chromosomes. Similar results were observed in two separate experiments. **C,** homozygous Elongin A-deficient ES cell with 80 chromosomes (4N) (left panel) or 160 chromosomes (8N) (right panel).

required for one or more genes involved in progression through the G2/M boundary of the cell cycle, although our initial study has not yet identified such genes. The other possibility is that there might be a functional link between transcription elongation by Elongin A and DNA recombination, as was found in *Saccharomyces cerevisiae* transcription elongation factors, HPR1 and THO2 (31–33). Mutations in these two yeast genes have been known to induce a hyper-recombination phenotype, which perhaps results from recruitment of the recombination machinery to the vicinity of inappropriately paused RNA polymerase II elongation complexes. If this is also the case in mammalian Elongin A, lack of functional Elongin A may induce a hyper-recombination phenotype and genomic instability, which could result in mitosis being delayed by the G2 checkpoint mechanism, although we presently do not have direct evidence of physical interaction between Elongin A and recombination proteins. However, it is also possible that Elongin A carries out some other cellular functions unrelated to transcription elongation *in vivo*. In fact, Elongin A has recently been shown to form a stable complex with the known components of the ubiquitin ligase, Cul5 and Rbx1 (34). Although we
presently do not know whether this complex actually possesses ubiquitin ligase activity, an intriguing possibility is that Elongin A together with Cul5 and Rbx1 mediate the degradation of factors, such as inhibitors of the mitosis promoting factor and positive regulators of the initiation of DNA replication, by a ubiquitin-proteasome pathway. Thus, Elongin A may function in the cell cycle by a mechanism that does not operate directly through a transcription pathway.

In summary, our results demonstrate that mammalian Elongin A is essential for transcription of a subset but not all of genes and is important for proper cell cycle progression. Further in vitro experiments will be necessary to understand the precise molecular mechanisms by which loss of functional Elongin A results in delayed onset of mitosis.

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