The human ELL gene, which is a frequent target for translocation in acute myeloid leukemia, was initially isolated from rat liver nuclei and found to be an RNA polymerase II elongation factor. Based on homology to ELL, we later cloned ELL2 and demonstrated that it can also increase the catalytic rate of transcription elongation by RNA polymerase II. To better understand the role of ELL proteins in the regulation of transcription by RNA polymerase II, we have initiated a search for proteins related to ELLs. In this report, we describe the molecular cloning, expression, and characterization of ELL3, a novel RNA polymerase II elongation factor approximately 50% similar to both ELL and ELL2. Our transcriptional studies have demonstrated that ELL3 can also increase the catalytic rate of transcription elongation by RNA polymerase II. The C-terminal domain of ELL, which we recently demonstrated to be required and sufficient for the immortalization of myeloid progenitor cells, shares strong similarities to the C-terminal domain of ELL3. ELL3 was localized by immunofluorescence to the nucleus of cells, and Northern analysis indicated that ELL3 is a testis-specific RNA polymerase II elongation factor.

Many cellular factors involved in human oncogenesis have been identified as genes at breakpoints of frequently occurring chromosomal translocations. The protein products of some of these genes are transcriptional factors that regulate the general or specific expression of many genes. The ELL gene was initially identified on chromosome 19p13.1, which undergoes frequent translocation with the trithorax-like MLL (ALL-1, HRX) gene on chromosome 11q23 in acute myeloid leukemia (1, 2). ELL is a 621 amino acid-containing protein that can increase the catalytic rate of transcription elongation of RNA polymerase II by suppressing transient pausing at multiple sites along the DNA from both promoter-dependent and promoter-independent templates (3–5). To date, eight elongation factors have been defined biochemically (17, 24). These factors are named SII (6–9), P-TPEf (10–11), DSIF (11–13), factor 2 (11, 14), TFIIF (15), elongin (SIII) (16), ELL (3, 18), and ELL2 (19). These RNA polymerase II elongation factors fall into several functional classes. Some can prevent arrest, like P-TPEf and SII. Some can regulate the rate of transcription elongation through nucleosomes, such as FACT (20). Others operate to increase the catalytic rate of transcription elongation by altering the $K_{m}$ and/or the $v_{max}$ of the polymerase, such as TFIIF, elongin (SIII), the ELL complex, and ELL2 (4, 5).

In an effort to better understand how transcription elongation by RNA polymerase II is controlled under normal conditions and in disease states, we are attempting to reconstitute RNA polymerase II transcription elongation machinery in vitro. In so doing, we have now identified and cloned a novel ELL family member, ELL3, and characterized its biochemical role in regulating transcription elongation.

**MATERIALS AND METHODS**

**Cloning and Expression of ELL3—**Searches of GenBankTM identified a short expressed sequence tag (accession number AA527300) that exhibited homology to the 3′-end of the coding regions of human ELL and ELL2. This partial sequence of human ELL3 enabled us to design a gene-specific primer (5′-GTTTTTCTAGACTATTTGTCGTCGTCGTCTC3′) and 3′antisense primer (5′-GTGTGGATCTCTCATCATCAGCTCCCTCCTGTTTCATCC-3′) using DNA polymerase Tli (Promega) with proofreading ability. The construct for expression of histidine-tagged ELL3 in bacteria was prepared by introducing the ELL3 ORF containing PCR product into the SalI and BamHI sites of M13mp18 bacteriophage vector, which contains the complete pETT7 transcription-translation regions as well as the sequence encoding the His tag. Recombinant ELL3 protein was expressed in Escherichia coli and purified from guanidine-solubilized inclusion bodies as described previously (3). We were able to express ELL3 in E. coli in an insoluble form; however, we were not able to either produce soluble ELL3 or renature ELL3 in a soluble form when expressed in E. coli inclusion bodies. Therefore, we set to express ELL3 in mammalian cells. The construct for expression of N-terminal histidine-tagged and C-terminal FLAG-tagged ELL3 in 293 tissue culture cells was obtained by PCR amplification using ELL3 specific 5′ primer (5′-GGAGGTGTCGACATGGAGGAGCTCCATGAGCCTCTG-3′) and 3′antisense primer (5′-GTGTGGATCTCTCATCATCAGCTCCCTCCTGTTTCATCC-3′) and 3′antisense primer (5′-GTGTGGATCTCTCATCATCAGCTCCCTCCTGTTTCATCC-3′). The digested PCR product was introduced into the EcoRI and XbaI sites of the tetracycline-regulated pIRE mammalian expression vector (CLONTECH). One ug of pIRE-ELL3 expression vector and 500 ng of pRTK-Hyg selection vector (CLONTECH) were cotransfected into 6 × 10⁵ 293 cells cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 3 mM l-glutamine, 100 μg/ml penicillin/streptomycin, 100 μg/ml G418 sulfate by lipofection. 48 h after transfection,
cells were purified in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 3 mM t-glutamine, 100 μg/ml penicillin/streptomycin, 100 μg/ml G418 sulfate, 100μg/ml hygromycin B, and 1 μg/ml tetracycline. Individual clones were trypsinized using cloning cylinders and plated into individual flasks. To induce expression of ELL3, 6 x 10⁶ cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 3 mM t-glutamine, 100 μg/ml penicillin/ streptomycin, 100 μg/ml G418 sulfate, 100 μg/ml hygromycin B. After 48 h, cells were harvested in 300 ml AmSO₄ extraction buffer and lysed by sonication. ELL3 was purified from total cell extracts by affinity chromatography with anti-FLAG M2 affinity gel (Sigma). The anti-FLAG M2 affinity gel was washed three times with high salt buffer (25 mM HEPES, pH 7.6, 500 mM NaCl, 0.1% Nonidet P-40, and 1 mM dithiothreitol). Approximately 100 μl of gel was incubated in batch with 500 μl of cell extract and tumbled at 4 °C for 1 h. The gel was collected by low speed centrifugation for 30 s and washed three times by resuspension in 500 μl of high salt buffer followed by three washes in 500 μl of low salt buffer (25 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM dithiobisulphite). Bound proteins were eluted with 100 μl of elution buffer (100 μg/ml FLAG peptide in low salt buffer). Protein elutions were aliquoted and frozen at −70 °C until use. The ELL3 that was purified via this method was used in our biochemical analysis.

**Immunofluorescence Staining—**Poly-L-lysine (1 mg/ml)-coated coverslips were placed in six-well tissue culture plates. 1 x 10⁷ ELL3 293 cells were plated on the coverslips and grown to approximately 70% confluency in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 3 mM t-glutamine, 100 μg/ml penicillin/streptomycin, 100 μg/ml G418 sulfate, 100 μg/ml hygromycin B, with or without 1 μg/ml tetracycline. Coverslips were washed briefly three times with 1× PBS and fixed with 3% paraformaldehyde in PBS followed by methanol for 6 min at −20 °C. Slides were stained with M2 FLAG monoclonal antibody (Sigma) (1:1000) in 1× PBS, 1% bovine serum albumin, 0.1% sodium azide for 45 min at 37 °C followed by staining with rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch) (1:1000) in 1× PBS, 1% sodium azide for 30 min at 28 °C, 100 μM nonradioactive CTP was added to the reaction mixture, and short transcripts were chased in the absence or presence of affinity-purified ELL3 purified from 293 cells or purified protein from mock-transfected 293 cells for the times indicated. Transcripts were analyzed by electrophoresis through a 6% polyacrylamide, 7 M urea gel and developed using a Molecular Dynamics, Inc. (Sunnyvale, CA) Phosphorimage instrument.

**Northern Blot Analysis—**Nitrocellulose filters containing approximately 2 mg of poly(A)⁺ RNA/lane from 16 different adult human tissues (CLONTECH) were used for Northern analysis. Filters were prehybridized and hybridized in 50% deionized formamide, 5% SSPE, 0.5% SDS, and 100 mg/ml denatured salmon sperm DNA at 42 °C. Blots were hybridized with 32P-labeled full-length ELL3 probe or β-actin cDNA probes for 16 h at 42 °C. The filters were washed twice in 2x SSC and 0.1% SDS for 30 min at 45 °C.

**RESULTS**

**Identification of Human ELL3—**A search of the GenBank® expressed sequence tag data base identified multiple short expressed sequence tag clones that had great identity to the C-terminal domains of ELL and ELL2. Since we recently demonstrated that this domain of ELL is required for the immortalization of myeloid progenitors by MLL-ELL found in human leukemia,² we set out to identify this full-length protein. Employing rapid amplification of cDNA ends-PCR and library hybridization methods, we obtained the ORF encoding ELL3. An approximately 1.2-kb DNA fragment containing the entire predicted ELL3 ORF was obtained by PCR using DNA polymerase Tli (Promega) with proofreading ability, and several clones were sequenced. The ELL3 ORF encodes an approximately 400-amino acid protein with an apparent molecular mass of about 50 kDa. As determined by the BESTFIT program of the Genetics Computer Group (GCG, Madison, WI) package (21), ELL3 has about 50% identity with ELL and ELL2 throughout its ORF (Fig. 1).

**Expression of ELL3 in Both Bacterial and Mammalian Cells and the Biochemical Analysis of the Recombinant Protein for Transcriptional Elongation Activity—**In our previous studies, we demonstrated that both ELL and ELL2 are capable of stimulating the overall rate of RNA chain elongation by RNA polymerase II by suppressing transient pausing along the DNA template (19, 22). Our structure/function studies demonstrated

² DiMartino, J. F., Miller, T., Aytton, P., Landewe, T., Hess, J. L., Cleary, M. L., and Shilatifard, A. (2000) Blood, in press.
that the elongation activation domain of ELL and ELL2 lies within the N-terminal 150 amino acids (19, 22). Since ELL3 demonstrated regions of high homology in its N terminus compared with ELL and ELL2, we set out to determine if ELL3 can also increase the catalytic rate of transcription elongation by RNA polymerase II. As shown in Fig. 2C, ELL3 is an RNA polymerase II elongation factor with functional properties similar to those reported for ELL and ELL2 (3, 19). The ability of ELL3 to increase the catalytic rate of transcription elongation was tested employing transcription reactions initiated from promoters and the basal transcription machinery. Briefly, preinitiation complexes were assembled by preincubation of purified RNA polymerase II, recombinant TBP, recombinant TFIIB, recombinant TFIIE, recombinant TFIIF, and purified TFIIH with DNA template containing AdML promoter. Highly radioactive transcripts were synthesized during a brief pulse carried out in the presence of ATP, GTP, UTP, and a limiting concentration of [$\alpha$-32P]CTP. These short promoter-specific transcripts were then elongated into full-length run-off transcripts in the presence of either purified protein from mock-transfected 293 cells (Fig. 2C, lanes 1–4) or purified ELL3 from 293 cells (Fig. 2C, lanes 5–8) and excess nonradioactive CTP. Transcripts were analyzed by electrophoresis through a 6% polyacrylamide, 7.0 M urea gel electrophoresis and developed with a Molecular Dynamics PhosphorImager instrument. As shown in Fig. 2C, transcripts synthesized in the presence of ELL3 (lanes 5–8) were substantially longer than those synthesized in its absence (lanes 1–4).

Analysis of the Expression Pattern of ELL3 in Human Tissue by Northern Analysis—To investigate the expression pattern of ELL3 in human tissue, Northern blotss containing poly(A)+ RNA from various human tissues were hybridized with a full-length ELL3-specific probe. As shown in Fig. 3, only RNA from the testis showed a single band at approximately 50 kDa, in agreement with the predicted size of the protein based on amino acid composition.

We next sought to determine if ELL3 could increase the catalytic rate of transcription elongation by RNA polymerase II. As shown in Fig. 2C, ELL3 is an RNA polymerase II elongation factor with functional properties similar to those reported for ELL and ELL2 (3, 19). The ability of ELL3 to increase the catalytic rate of transcription elongation was tested employing transcription reactions initiated from promoters and the basal transcription machinery. Briefly, preinitiation complexes were assembled by preincubation of purified RNA polymerase II, recombinant TBP, recombinant TFIIB, recombinant TFIIE, recombinant TFIIF, and purified TFIIH with DNA template containing AdML promoter. Highly radioactive transcripts were synthesized during a brief pulse carried out in the presence of ATP, GTP, UTP, and a limiting concentration of [$\alpha$-32P]CTP. These short promoter-specific transcripts were then elongated into full-length run-off transcripts in the presence of either purified protein from mock-transfected 293 cells (Fig. 2C, lanes 1–4) or purified ELL3 from 293 cells (Fig. 2C, lanes 5–8) and excess nonradioactive CTP. Transcripts were analyzed by electrophoresis through a 6% polyacrylamide, 7.0 M urea gel and developed using a Molecular Dynamics PhosphorImager instrument. As shown in Fig. 2C, transcripts synthesized in the presence of ELL3 (lanes 5–8) were substantially longer than those synthesized in its absence (lanes 1–4).

Analysis of the Expression Pattern of ELL3 in Human Tissue by Northern Analysis—To investigate the expression pattern of ELL3 in human tissue, Northern blots containing poly(A)+ RNA from various human tissues were hybridized with a full-length ELL3-specific probe. As shown in Fig. 3, only RNA from the testis showed a single band at approximately 2-kb hybridizing with human ELL3 probe. In the liver and pancreas mRNA (data not shown). The blots were stripped and reprobed with a human actin cDNA probe to ensure equivalent mRNA loading.

Nuclear Localization of ELL3—We developed stable cell lines regulated for the expression of ELL3 as described under “Materials and Methods.” We constructed the FLAG-ELL3 cDNA under the minimal cytomegalovirus promoter regulated
by the tetracycline operator and developed ELL3 293 Tet cell lines expressing full-length ELL3 under control of the tetracycline-regulated promoter. Removal of tetracycline from the tissue culture medium for 24 h resulted in the expression of full-length ELL3 of approximately 50 kDa as determined by Western blot using FLAG monoclonal antibody (data not shown). To determine the subcellular localization of ELL3, we employed FLAG-ELL3 Tet Off cell lines and FLAG monoclonal antibody. As shown in Fig. 4A, ELL3 expression off and stained for ELL3. B, ELL3 expression on and stained with DAPI to visualize DNA. C, ELL3 expression on and stained for ELL3.

**DISCUSSION**

We report here the identification, expression, and biochemical analysis of ELL3, a novel RNA polymerase II elongation factor that is specifically expressed in human testis. ELL3 is an approximately 400 amino acid-containing protein that has approximately 50% sequence identity to both ELL and ELL2 proteins (Fig. 1). Like ELL and ELL2, ELL3 is also capable of increasing the catalytic rate of transcription elongation catalyzed by RNA polymerase II initiated from promoters and the basal transcription machinery. Our immunofluorescence studies have indicated that ELL3 is localized in the nucleus of mammalian cells. We note that proteins expressed under the transient transfection conditions are extremely overexpressed in cells and may result in the incorrect interpretation of the localization data. For this reason, we have developed an ELL3-stable cell line (which contains about 1 copy of ELL3/cell) and have demonstrated that when ELL3 is expressed in these cells, all of the ELL3 in all of the cells expressing ELL3 is found within the nucleus. We find ELL3 diffused evenly within the nucleus, a common characteristic of most general transcriptional elongation factors (23). This method of localization has also been used for both ELL and ELL2 and also the MLL protein.

We have recently demonstrated that the C-terminal domain of ELL is required and sufficient for the immortalization of myeloid progenitors by the MLL-ELL fusion protein found in patients with acute myeloid leukemia. Our data also indicated that the presence of the elongation activity of ELL can increase the number of immortalized cells after the third round of passage. Very little is known about the role of the C-terminal domain of ELL and how it may function in biological systems. Since both ELL2 and ELL3 have strong conservation of their C-terminal domain and exhibit a great degree of homology to the C-terminal domain of ELL, we can speculate that this domain of the ELLs plays a pivotal physiological role.

It has been demonstrated that expression of ELL in RAT1 cells leads to increased colony formation, which is coincident with expression of the AP-1 protein, c-Fos (5). Interestingly, these two functions depend on the presence of a lysine-rich region within the C terminus present in both ELL1 and ELL2 but not entirely present in ELL3. Whether ELL2 and/or ELL3 have similar oncogenic properties to those apparently mediated by ELL remains to be determined.

We also note that a homology search of the GenBank™ data base has revealed that the conserved C-terminal domain of the ELLs bears a striking resemblance to the ZO-1 binding domain of occludin (26, 27). This similarity of the C-terminal domain of ELL to the ZO-1 binding domain of occludin is about 42% for ELL2, 44% for ELL3, and 40% for ELL. The ZO-1 protein is a member of the family of membrane-associated guanylate kinase homologs that is thought to be important for signal transduction (28). The ZO-1 protein, which is predominantly found in the cytosol of contact-inhibited cultured cells, was recently demonstrated to translocate to the nucleus of subconfluent cells (29). This indicates that ZO-1 may be involved in signaling pathways controlled by cell-cell contact. Whether the conserved C-terminal domain of the ELLs can interact with either ZO-1 or

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Fig. 4. Immunofluorescent localization of ELL3. 1 × 10⁶ clonal ELL3 293 cells were plated on coverslips in six-well tissue culture plates and grown to approximately 70% confluence in the absence or presence of 1 μg/ml tetracycline to regulate ELL3’s expression. Cells were fixed as described under “Materials and Methods” and stained with M2 FLAG monoclonal antibody (1:1000) in 1× PBS followed by staining with rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (1:1000) in 1× PBS, 1 μg/ml DAPI. After mounting, slides were photographed with an Olympus microscope. A, ELL3 expression off and stained for ELL3. B, ELL3 expression on and stained with DAPI to visualize DNA. C, ELL3 expression on and stained for ELL3.

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3 T. Landewe and A. Shilatifard, unpublished data.
ZO-1-like proteins or with proteins that interact with ZO-1 or ZO-1-like proteins is currently unknown. However, it is feasible to speculate that the ELLs can regulate transcription via a signal transduction pathway involving the ZO-1 or ZO-1-like protein(s).

Studies of the functional interaction of ELL have demonstrated that ELL is capable of interaction with p53 (2, 33). This interaction of ELL with p53 results in the regulation of transcriptional activities of both ELL and p53 in vitro (33). Structure/function studies have demonstrated that functional interaction of ELL with p53 requires the N-terminal half of ELL protein. We have tested if both ELL2 and ELL3 interact with p53. Our functional interaction studies indicate that ELL2 and ELL3 are both capable of physical interaction with p53 and that this interaction requires the C-terminal domain of p53 (data not shown). The investigation of the physiological role for such interactions between the ELLs with p53 is under way in our laboratories.

Our Northern analysis which was probed with a full-length ELL3 probe has demonstrated that ELL3 is testis-specific. This is in contrast to ELL, which is ubiquitously expressed, and ELL2 which is expressed in most tissues tested except for the kidneys (19). There are other testis-specific RNA polymerase II elongation factors such as SII and elongin A2 (7, 30, 31). What is the function of these testis elongation factors such as SII and elongin A2? What is in contrast to ELL, which is ubiquitously expressed, and ELL3 probe has demonstrated that ELL3 is testis-specific. This ELL3 are both capable of physical interaction with p53 and that this interaction requires the C-terminal domain of p53 (data not shown). The investigation of the physiological role for such interactions between the ELLs with p53 is under way in our laboratories.

Our Northern analysis which was probed with a full-length ELL3 probe has demonstrated that ELL3 is testis-specific. This is in contrast to ELL, which is ubiquitously expressed, and ELL2 which is expressed in most tissues tested except for the kidneys (19). There are other testis-specific RNA polymerase II elongation factors such as SII and elongin A2 (7, 30, 31). What is the function of these testis elongation factors in vivo? The data presented, together with other reports that have shown a dramatic increase of RNA polymerase II and several other general transcription initiation factors during late spermatogenesis in rodents (32) and also the lack of the expression of ELL3 in ovaries, suggest that ELL3 may play an important role in the process of spermatogenesis. However, the expression of ELL3 is not detectable in ovaries; therefore, ELL3 may not play any general role in meiosis. Currently, we are pursuing the cell type specificity of ELL3 and plan to generate ELL3-deficient mice to determine the role of this protein in vivo. Finally, the ability of the ELLs to regulate the rate of messenger RNA synthesis, catalyzed by RNA polymerase II, makes the study of the mechanism of action of these proteins extremely important in understanding the mechanism of regulation of mammalian gene expression.

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Note Added in Proof—While our manuscript was in proof, a search of the newly released database with ELL3 cDNA indicated that the gene encoding for human ELL3 is located on chromosome 15q15.

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