Identification and Purification of the Holo-ELL Complex

EVIDENCE FOR THE PRESENCE OF ELL-ASSOCIATED PROTEINS THAT SUPPRESS THE TRANSCRIPTIONAL INHIBITORY ACTIVITY OF ELL*

(Received for publication, February 12, 1998, and in revised form, March 4, 1998)

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The human ELL gene on chromosome 19 undergoes frequent translocation with the trithorax-like MLL gene on chromosome 11 in acute myeloid leukemia. Recently, it was demonstrated that the product of the human ELL gene encodes an RNA polymerase II elongation factor (Shilatifard, A., Lane, W. S., Jackson, K. W., Conaway, R. C., and Conaway, J. W. (1997) J. Biol. Chem. 272, 22355–22363). Here, we report the identification and purification of a large ELL-containing complex that contains three proteins in addition to ELL and that we have named the Holo-ELL complex. The Holo-ELL complex can increase the catalytic rate of transcription elongation by RNA polymerase II. However, unlike the ELL polypeptide alone, the Holo-ELL complex is not capable of negatively regulating polymerase activity in promoter-specific transcription in vitro. The inability of the Holo-ELL complex to negatively regulate polymerase activity in promoter-specific transcription suggests that one or more of the ELL-associated proteins regulate this activity, possibly through an interaction with the N-terminal domain of the ELL protein, which was shown to be required for the transcriptional inhibitory activity of ELL. Characterization of these ELL interacting proteins should help define the regulation of the biochemical activities of ELL and how loss of this regulation leads to the development of acute myeloid leukemia.

The human ELL gene was initially identified as a gene that undergoes frequent translocations with the trithorax-like MLL gene in acute myeloid leukemia (AML) (1, 2).† We demonstrated that ELL, a basic 621-amino acid protein, interacts directly with Pol II (3, 4) and can increase the catalytic rate of transcription elongation by Pol II by suppressing transient pausing at multiple sites along the DNA (5). ELL can also regulate the transcription by a novel Pol II interaction domain that is capable of negatively regulating polymerase activity in promoter-specific transcription initiation in vitro (3). Remarkably, the MLL-ELL translocation, which is found in patients with AML, results in the deletion of a portion of this functional domain (1, 2). ELL mutants lacking the sequence that is deleted by the translocation are fully active in elongation and can interact with Pol II. However, such mutants fail to inhibit initiation by Pol II (3). It is not yet clear how the transcription initiation inhibitory activity of ELL normally plays a role in the regulation of gene expression or the regulation of the cell cycle. However, further studies on the biochemical mechanism and physiological function of this functional domain of ELL and proteins that interact with this domain will improve our understanding of the role of this domain of the ELL protein in transcriptional regulation and in development of leukemia.

The original purification of ELL took advantage of hydrophobic interactions and reverse-phase chromatography to purify a single polypeptide that was active as an RNA polymerase II elongation factor (4). This purification employed denaturing solvents and conditions that destroy multiprotein complexes, and the proteins associated with ELL were not identified initially. However, alternate methods of purification procedures demonstrated that ELL exists as a multiprotein complex in vivo. As part of our effort to understand how elongation by RNA polymerase II is normally controlled and how it is dysregulated in certain malignancies, we set out to purify this ELL-containing complex. In this report, we describe the purification and characterization of a novel ELL-containing complex that we have named the Holo-ELL complex.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure ribonucleoside 5′-triphosphates were purchased from Amersham Pharmacia Biotech. [α-32P]CTP was obtained from Amersham. Leupeptin, antipain, phenylmethylsulfonyl fluoride, and heparin were obtained from Sigma. Bovine serum albumin (Pentex fraction V) and Western development reagents were obtained from ICN ImmunoBiologicals. Glycerol (spectranalyzed grade), potassium chloride, Hepes, Tris, Ammonium Sulfate, and ultrapure sucrose were purchased from Fisher. The chromatographic columns DEAE-5PW and SP-5PW were purchased from Tosohas, and Mono-Q, Mono-S, Suprose-12 HR, Suprose-6 PC, and Mini-Q were purchased from Amersham Pharmacia Biotech.

Step I: Purification of the Holo-ELL Complex—Rat liver extract was prepared by homogenization of 180 rat livers as described previously (4), with the exception that nuclear and cytosolic extract were pooled together after the removal of the lysosomal fraction. The purification of the holo-ELL was performed at 4 °C, and the fractions were not frozen unless indicated.

Step II: DEAE-cellulose—The 0–40% (NH4)2SO4 fraction was dialyzed to a conductivity equivalent to that of 100 mM (NH4)2SO4 in buffer A (40 mM Tris-HCl, pH 7.9, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) and was then mixed with 1.0 liter of DEAE-cellulose equilibrated to a conductivity equivalent to that of 100 mM (NH4)2SO4 in buffer A.
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**Rat Liver Extract**

- 0–40% (NH₄)₂SO₄ (I)
- DEAE-52 (II)
- P-Cel (III)
- HW 65F (IV)
- DEAE-52 (V)
- DEAE-5PW (VI)
- SP-5PW (VII)
- Mono-Q HR (VIII)
- Superose-12 (IX)
- Mini-Q (X)
- Superose-6 PC (XI)

**Holo-ELL**

- DEAE-cellulose—Fraction IV was dialyzed against buffer A containing 70 mM KCl and was then mixed with 100 ml of DEAE-cellulose equilibrated with buffer A containing 70 mM KCl. The slurry was allowed to set for an hour and then filtered at 300 ml/h. The DEAE-cellulose column was washed at the same flow rate with buffer A containing 70 mM KCl until the eluate contained < 0.10 mg/ml protein. The column was then eluted with buffer A containing 300 mM KCl, 50-ml fractions were collected, and protein-containing fractions were pooled (fraction V).

**Step VI: TSK DEAE-5PW**—Fraction V was dialed against buffer A containing 70 mM KCl and then centrifuged at 20,000 g for 30 min and applied to a 50-ml TSK DEAE-5PW (Tosoh) equilibrated in buffer A containing 50 mM KCl. Transcription elongation activity was eluted at 5 ml/min with a 500-ml linear gradient from 50 to 500 mM KCl. 10-ml fractions were collected, and the transcriptionally active fractions were collected (fraction VI).

**Step V: TSK DEAE-5PW**—Fraction IV was dialyzed against buffer A containing 70 mM KCl and then centrifuged at 20,000 g for 30 min and applied to a 50-ml TSK DEAE-5PW (Tosoh) equilibrated in buffer A containing 50 mM KCl. Transcription elongation activity was eluted at 5 ml/min with a 500-ml linear gradient from 50 to 500 mM KCl. 10-ml fractions were collected, and the transcriptionally active fractions were collected (fraction VI).

**Step IV: Size Exclusion Chromatography**—Solid (NH₄)₂SO₄ (0.4 mg/ml) was slowly added to fraction III with stirring. 0.1 μl of 10 N NaOH/g of (NH₄)₂SO₄ was then added, and the suspension was stirred for an additional 60 min. The (NH₄)₂SO₄ precipitate was collected by centrifugation at 10,000 × g for 60 min and dissolved to a final volume of 7 ml in buffer C. The solution was dialed against buffer C (40 mM Hepes-NaOH, pH 7.4, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) until the conductivity was equivalent to that of buffer C in 400 mM KCl. The resulting supernatant was applied to HW 65F (Tosoh) size exclusion column equilibrated in buffer C containing 400 mM KCl.

**Step III: Phosphocellulose**—Fraction II was loaded onto a 300-ml phosphocellulose column (P 11, Whatmann) equilibrated in buffer B (40 mM Hepes-NaOH), pH 7.9, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF containing 100 mM KCl. The column was eluted stepwise at three packed column volume/h with buffer B containing 500 mM KCl. 10% column volume fractions were collected, and the protein-containing fractions were pooled (fraction III).

**Step II: DEAE-cellulose**—The slurry was allowed to sit for an hour and then filtered at 3.0 liters/h. The DEAE-cellulose column was washed at the same flow rate with buffer A containing 100 mM (NH₄)₂SO₄ until the eluate contained 0.10 mg/ml protein. The fractions containing protein were pooled and dialyzed against buffer B with 100 mM KCl (fraction II).

**Identification of a large ELL-containing complex.** Either recombinant ELL (A) or rat liver extract (B) were subjected to gel filtration chromatography. The presence of the ELL polypeptide was determined by Western analysis with monoclonal antibodies to ELL. The transcriptional elongation activity of ELL was determined by testing the ability of each fraction to increase the rate of the synthesis of 135-nucleotide (nt) transcript from the T-less cassette of the oligo(dC)tailed template of pCpGR220 as described before. The elution profile of the molecular mass standards is indicated by arrows.

**FIG. 1.** Identification of a large ELL-containing complex. Either recombinant ELL (A) or rat liver extract (B) were subjected to gel filtration chromatography. The presence of the ELL polypeptide was determined by Western analysis with monoclonal antibodies to ELL. The transcriptional elongation activity of ELL was determined by testing the ability of each fraction to increase the rate of the synthesis of 135-nucleotide (nt) transcript from the T-less cassette of the oligo(dC)tailed template of pCpGR220 as described before. The elution profile of the molecular mass standards is indicated by arrows.

**FIG. 2.** Purification of the Holo-ELL complex. 0–40% (NH₄)₂SO₄ fraction from rat liver extract was subjected to chromatography following this purification scheme. P-cell, phosphocellulose P-11; DEAE-5PW, Bio-Gel TSK DEAE-5-PW; SP-5-PW, Bio-Gel TSK SP-5-PW.
of transcriptionally active fractions were pooled (fraction VIII). Fraction VIII was dialyzed to a conductivity equivalent to that of 70 mM KCl in buffer A, was centrifuged at 20,000 x g for 30 min, and then was applied to a Mono-Q HR column (Amersham Pharmacia Biotech) equilibrated in buffer A containing 50 mM KCl. The column was eluted at 50 ml/min with a 10-ml linear gradient from 50 mM to 500 mM KCl. 1-ml fractions were collected, and the transcriptionally active fractions were pooled (fraction IX).

Step IX: Size Exclusion Chromatography on Superose 12 HR—The conductivity of fraction VIII was adjusted to a conductivity equivalent to that of 400 mM KCl in buffer C by dropwise addition of buffer C containing 1 x KCl. Fraction VIII was centrifuged at 14,000 x g for 30 min and then applied to a Superose-12 HR column (Amersham Pharmacia Biotech) equilibrated in buffer C containing 400 mM KCl. The column was eluted at 0.5 ml/min, and 1-ml fractions were collected. The transcriptionally active fractions were pooled and dialyzed against buffer A containing 50 mM KCl (fraction X).

Step X: Mini-Q—Fraction IX was centrifuged at 14,000 x g for 30 min and then applied to a Mini-Q column (Amersham Pharmacia Biotech) equilibrated in buffer A containing 50 mM KCl. Transcription elongation activity was eluted at 100 µl/min with a 1-ml linear gradient from 50 to 500 mM KCl. Fractions of 150 µl were collected, and the transcriptionally active fractions were pooled (fraction X).

Step XI: Size Exclusion Chromatography on Superose 6 PC—The conductivity of fraction X was adjusted to a conductivity equivalent to that of 400 mM KCl in buffer C by dropwise addition of buffer C containing 1 x KCl. Fraction X was centrifuged at 14,000 x g for 30 min and then applied to a Superose 6 PC column (Amersham Pharmacia Biotech) equilibrated in buffer C containing 400 mM KCl. The column was eluted at 50 µl/min, and 50-µl fractions were collected. Small aliquots were made, and fractions were frozen at −80°C.

Assays of Transcription Elongation—Transcription elongation was assayed from both PGR220 S/P/X tailed template and AdML promoter as described before (4).

RESULTS

Evidence for the Presence of the Holo-ELL Complex—Since the discovery of ELL as an RNA polymerase II elongation factor (4), we have obtained evidence that ELL exists in a stable complex with other cellular proteins. The recombinant ELL is transcriptionally active as a single subunit protein with a relatively molecular mass of about 80 kDa on SDS-PAGE and ~70 kDa on gel filtration chromatography (Fig. 1A). However, the analysis of crude rat liver extracts on gel filtration chromatography indicates that the transcription elongation activity was present in a large transcriptionally active multiprotein complex (molecular mass of 210 kDa) that contains ELL (Fig. 1B). This was shown with monoclonal antibodies specific to the ELL protein.

Comparative studies of the activation of transcription elongation with the partially purified ELL from rat liver nuclear extract and recombinant ELL demonstrated that both forms exhibited similar transcriptional elongation activity on linear templates. However, only the partially purified native ELL demonstrated transcription elongation activity from circular templates. This difference suggested that ELL-associated proteins might regulate the transcriptional activity of ELL.

Purification of the Holo-ELL Complex—The purification of the Holo-ELL from total liver extract is summarized in Fig. 2 and Table I. The elongation stimulatory activity assay used to purify the Holo-ELL measured its ability to stimulate the rate of accumulation of 135-nucleotide transcripts synthesized by RNA polymerase II in the T-less cassette of the oligo(dC)-tailed template pCpGR220 S/P/X (Fig. 3C) (4, 6). Analysis of fractions from size exclusion chromatography (step XI, Fig. 2) by SDS-PAGE revealed that the transcriptional elongation activity of the Holo-ELL copurified with four polypeptides of ~20, 30, 45, and 80 kDa, which were named EAP20, EAP30, EAP45, and EAP80, respectively (Fig. 3D). The solution molecular mass of this ELL-containing complex was estimated to be 210 kDa from the log of molecular mass of standard proteins versus their pattern of elution (Fig. 3D).

Analysis of EAP80 with MALDI-MS and Edman Sequencing—The recombinant ELL polypeptide has an apparent molecular mass of ~80 kDa on SDS-PAGE. The EAP80 from the SDS-PAGE was further analyzed by both matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and tryptic peptide sequencing (7, 8). The MALDI-MS analysis of EAP80 indicated that EAP80 is the ELL polypeptide with a 100% probability score (data not shown). The sequencing analysis of EAP80 tryptic peptide showed a sequence that exactly matched amino acids 394–406 of the ELL sequence (Fig. 4A). Both the MALDI-MS and the sequencing of the tryptic peptide obtained from EAP80 demonstrated that EAP80 is the ELL polypeptide.

Several findings here are consistent with the notion that ELL actually is in a complex with EAP20, EAP30, and EAP45: (i) the elongation activity copurifies with a complex containing 80, 40, 30-, and 20-kDa polypeptides both on ion exchange or size exclusion chromatography; (ii) a monoclonal antibody raised against ELL was able to co-immunoprecipitate all EAPs with ELL (EAP80) when the complex from step X was used. When the same experiment was performed with monoclonal
antibodies raised against ELL2, which does not immunoprecipitated ELL, no EAP was co-immunoprecipitated (data not shown); and (iii) SDS-polyacrylamide gel electrophoresis demonstrated that Holo-ELL polypeptides (EAP80, EAP40, EAP30, and EAP20) are present in roughly equimolar amounts and account for more than 90% of the protein in the most highly purified Holo-ELL preparations (Fig. 3).

The Purified Holo-ELL Complex Can Increase the Rate of Transcription Elongation by RNA Polymerase II—First we examined the ability of the purified Holo-ELL complex to increase the catalytic rate of transcription elongation from promoter-independent oligo(dC)-tailed template pCpGR220. Preinitiation complexes were assembled at the AdML promoter with recombinant TBP, TFIIH, TFIIE, TFIIF, and purified Pol II and TFIIH. Transcription was initiated by the addition of 50 μM ATP, 50 μM GTP, 2 μM UTP, 10 μCi of [α-32P]CTP, and 7 mM MgCl2. After 10 min, 100 μM non-radioactive CTP was added to reaction mixtures. Where indicated 1 μl of the Holo-ELL complex containing about 100 ng of EAP80 was added to the reaction mixtures, which were then incubated for further times as indicated. Transcripts were analyzed by electrophoresis through a 7% polyacrylamide, 7 M urea gel.

**Fig. 4.** Sequence similarity of tryptic fragment of EAP80 and the human ELL protein and transcriptional elongation activity of the Holo-ELL form promoter-specific template. A, EAP80 was digested with trypsin and fractionated by microbore high pressure liquid chromatography. Optimal peptides were digested by MALDI-MS and sequenced with automated Edman degradation. Capital letters indicate the highest probability sequence. B, effect of the Holo-ELL (from step X) on the kinetics of promoter-dependent transcription by RNA polymerase II was determined. Preinitiation complexes were assembled at the AdML promoter with recombinant TBP, TFIIH, TFIIE, TFIIF, and purified Pol II and TFIIH. Transcription was initiated by the addition of 50 μM ATP, 50 μM GTP, 2 μM UTP, 10 μCi of [α-32P]CTP, and 7 mM MgCl2. After 10 min, 100 μM non-radioactive CTP was added to reaction mixtures. Where indicated 1 μl of the Holo-ELL complex containing about 100 ng of EAP80 was added to the reaction mixtures, which were then incubated for further times as indicated. Transcripts were analyzed by electrophoresis through a 7% polyacrylamide, 7 M urea gel.
addition of UTP (final concentration, 2 μM) and a large excess of nonradioactive CTP. At the conclusion of the reactions, transcripts synthesized in the presence of the Holo-ELL complex were considerably longer than the transcripts synthesized in its absence (data not shown). This indicates that the Holo-ELL complex is capable of increasing the rate of transcription elongation by RNA polymerase II in the absence of initiation factors, which is also shown in both Figs. 1B and 3C.

We next tested whether the Holo-ELL complex could increase the rate of transcription from the AdML promoter in the presence of general initiation factors. Briefly, preinitiation complexes were assembled by preincubation of purified RNA polymerase II, TBP, TFIIH, TFIIIE, and TFIIH with DNA template containing AdML promoter. After a short time, highly radioactive transcripts were synthesized during a brief pulse carried out in the presence of ATP, GTP, UTP, and a limiting concentration of [α-32P]CTP. These short promoter-specific transcripts were then elongated into full-length run-off transcripts in the presence or absence of the Holo-ELL complex (from step XI) and excess of nonradioactive CTP. As shown in Fig. 4B, comparison of the kinetics of accumulation of full-length run-off transcripts reveals that the Holo-ELL can also stimulate the rate of transcription elongation of promoter-specific transcription by RNA polymerase II.

The Purified Holo-ELL Complex Is Unable to Negatively Regulate Polymerase Activity in Promoter-Specific Transcription—Next we tested the ability of this complex to negatively regulate polymerase activity in promoter-specific transcription, a property of ELL alone (3). As demonstrated previously, RNA polymerase II and the general initiation factors will synthesize the trinucleotide CpApC at the AdML promoter when provided with CpA and [α-32P]CTP (Fig. 5A). The addition of recombinant ELL to the transcription reactions before the formation of preinitiation complex resulted in a substantial reduction in the yield of CpApC (Fig. 5B). To our surprise, the addition of the purified Holo-ELL complex to transcription reactions before assembly of the preinitiation complex did not decrease the yield of CpApC synthesis (Fig. 5C). This indicates that the transcriptional inhibitory activity of the ELL protein is suppressed in the Holo-ELL complex.

**DISCUSSION**

ELL was discovered as a novel transcription elongation factor that undergoes frequent translocations in AML (1–4). The original purification of ELL took advantage of chromatography systems that require the usage of denaturing solvents and conditions that can destroy multiprotein complexes. For this reason, the proteins associated with ELL were not identified initially. Here we report the identification and purification of ELL together with three other proteins that form the Holo-ELL complex. This ELL-containing complex was purified from crude rat liver extract to homogeneity following the purification scheme outlined in Fig. 2. The three other cellular proteins found in complex with ELL have molecular masses of 20, 30, and 45 kDa on SDS-PAGE. These proteins were named EAP20, EAP30, and EAP45, respectively (Fig. 3). The Holo-ELL complex was also shown to possess a native molecular mass of approximately 210 kDa on size exclusion chromatography (Fig. 3D). Unlike the ELL polypeptide, the Holo-ELL complex is not capable of negatively regulating polymerase activity in promoter-specific transcription in vitro.

The partner of ELL in acute myeloid leukemia is the product of the MLL gene, which encodes a large, multi-domain 3,968-amino acid protein. It contains a N-terminal A-T hook DNA-binding domain, a methyltransferase-like domain, and a C-terminal trithorax-like region composed of a transcriptional activation domain and several contiguous zinc fingers (9–11). Characterization of chromosomal abnormalities in a large number of human cancers revealed the MLL gene to be a recurring target for translocation in a variety of clinically distinct leukemias. Although nine other translocation partners of MLL have been cloned (10), ELL is the only partner whose function is known (4). The breakpoint of every MLL translocation creates a putative oncogene that encodes nearly the entire translocation partner fused to the N terminus domain of the MLL protein (12). The fact that all these translocations occur
within the same region of MLL but each is associated with a
clinically distinct form of leukemia suggests that the MLL
translocation partners, such as ELL, play a major role in de-
termining the leukemic phenotype. Recently, it was demon-
strated that the replacement of the normal MLL gene with an
MLL-AF9 chimera led to the development of leukemia in mice,
suggesting that translocation to an appropriate partner is the
cause for the development of AML (13).

Why ELL is involved in leukemia still a mystery. However,
ELL contains an N-terminal novel Pol II interaction domain
that is capable of negatively regulating polymerase activity in
promoter-specific transcription initiation in vitro (3). ELL de-
letions of the fifty N-terminal amino acids are fully active in
elongation and can interact with Pol II; however, such mutants
failed to inhibit initiation by Pol II (3). Remarkably, the MLL-
ELL translocation that is found in patients with AML results
in the deletion of exactly this functional domain (1, 2). The
discovery of ELL-associated proteins that can suppress its
transcriptional inhibitory activity suggests that one or more of
the ELL-associated proteins interacts or at least renders non-
functional the N-terminal domain of the ELL protein, which
were shown to be important for the transcriptional inhibitory
activity of ELL. In this view, the interaction of ELL with one of
the EAPs normally regulates the transcriptional inhibitory
activity of ELL via its inter-
acting proteins has shed new light on the regulation of tran-
scription by RNA polymerase II and its accessory factors and
provides a new hypothesis for why the MLL-ELL translocation
leads to the development of leukemia.

Acknowledgments—I am thankful to Dr. William S. Sly for encour-
age and advice during the course of this work and Dr. K. Williams
and Kathy Stone for MALDI-MS and sequence analysis of tryptic pep-
tide of EAP80.

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