Identification and Characterization of a *Schizosaccharomyces pombe* RNA Polymerase II Elongation Factor with Similarity to the Metazoan Transcription Factor ELL

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ELL family transcription factors activate the rate of transcript elongation by suppressing transient pausing by RNA polymerase II at many sites along the DNA. ELL-associated factors 1 and 2 (EAF1 and EAF2) bind stably to ELL family members and act as strong positive regulators of their transcription activities. Orthologs of ELL and EAF have been identified in metazoa, but it has been unclear whether such RNA polymerase II elongation factors are utilized in lower eukaryotes. Using bioinformatic and biochemical approaches, we have identified a new *Schizosaccharomyces pombe* RNA polymerase II elongation factor that is composed of two subunits designated SpELL and SpEAF, which share weak sequence similarity with members of the metazoa ELL and EAF families. Like mammalian ELL-EAF, SpELL-SpEAF stimulates RNA polymerase II transcription elongation and pyrophosphorolysis. In addition, like many yeast RNA polymerase II elongation factors, deletion of the SpELL gene renders *S. pombe* sensitive to the drug 6-azauracil. Finally, phylogenetic analyses suggest that the SpELL and SpEAF proteins are evolutionarily conserved in many fungi but not in *Saccharomyces cerevisiae*.

The human ELL gene was originally identified as a gene that undergoes translocations with the trithorax-like MLL gene in acute myeloid leukemia (1, 2). Subsequently, ELL was purified from rat liver nuclear extracts based on its ability to activate the rate of transcript elongation by RNA polymerase II (pol II) in *vitro*. Mechanistic studies revealed that ELL interacts directly with transcribing pol II in *vitro* and functions by suppressing transient pausing by the enzyme at many sites along the DNA (3, 4). Searches of sequence data bases identified two additional mammalian ELL family members, designated ELL2 and ELL3, as well as a single *Drosophila melanogaster* ortholog. All these proteins were shown to function similarly to activate the rate of elongation by pol II in *vitro* (5–7).

EAF1 and EAF2 are two closely related proteins that were first identified in yeast two-hybrid screens for ELL-interacting proteins (8, 9). In mammalian cells, EAF1, EAF2, and ELL are colocalized in Cajal bodies, nuclear structures that are enriched in factors involved in transcription and mRNA processing (10). Recently, we showed that EAF1 and EAF2 bind directly to ELL family members and function as strong positive regulators of ELL transcription activity in *vitro* (11).

Although little is known about the role of the ELL-EAF complex in transcriptional regulation in *vivo*, the *Drosophila* gene encoding the ELL homolog *Suppressor of Triplo-lethal* (*Su(Tpl)*) is essential for viability in flies. Some mutations in *Su(Tpl)* suppress lethality resulting from overexpression of the Tpl gene, perhaps by impairing synthesis of Tpl mRNA (12). The *Xenopus laevis* EAF2 protein functions during eye development to activate transcription of the gene encoding the essential Rx homeodomain transcription factor (13). Consistent with a role for ELL in controlling transcript elongation in *vivo*, *Drosophila* ELL colocalizes with pol II at transcriptionally active sites on polytene chromosomes, and evidence suggests that mutations in *Su(Tpl)* may preferentially affect synthesis of some long transcripts (7).

Many components of the pol II transcription machinery are highly conserved across species from mammals to yeast; however, until now attempts to identify orthologs of the ELL and EAF proteins in fungi have been unsuccessful, prompting speculation that elongation factors like ELL might have evolved only after the emergence of multicellular organisms, where genes can be many tens of kilobases long, and fine-tuning of the transcriptional program is expected to be particularly important for differentiation and development (12). In this work, we identify in fungi genes that encode proteins similar to ELL and EAF. These genes are found in *Schizosaccharomyces pombe* and several other fungi with completely sequenced genomes but not in *Saccharomyces cerevisiae*. Like their counterparts from larger
eukaryotes, *S. pombe* ELL and EAF (SpELL and SpEAF) interact with one another to form a stable heterodimer that potently activates transcription elongation by pol II *in vitro*. Like yeast strains bearing mutations in several other components of the pol II elongation machinery, *S. pombe* lacking the gene encoding ELL exhibit a 6-azauracil-sensitive phenotype, suggesting that ELL could play an important role in transcriptional regulation even in simple, unicellular organisms.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled ultrapure ribonucleoside 5′-triphosphates and [α-32P]CTP (400 mCi/mmol; 1 Ci = 37 GBq) were purchased from Amersham Biosciences. Recombinant RNasin ribonuclease inhibitor was obtained from Promega. Anti-FLAG (M2) monoclonal antibodies, anti-Myc (C-3956) rabbit polyclonal antibodies, anti-FLAG (M2)-agarose, and anti-FLAG peptide were purchased from Sigma. Light chain-specific anti-mouse antibodies were from Bethyl Laboratories and labeled with Alexa Fluor 680 (Invitrogen) according to the manufacturer’s instructions.

**Protein Sequence and Structure Analyses**—Data base searches were performed using the PSI-BLAST program (14) with the standard parameters, i.e. profile inclusion cutoff 0.002, SEG filter, and composition-based statistics invoked; −h 0.002, −F t, −T t. Multiple sequence alignments were done using the MUSCLE program (15). Fold recognition was done using the HHsearch program (16).

**Cloning of *S. pombe* ell1 and eaf1 Genes**—The SMART™ RACE cDNA amplification system (Clontech) was used according to manufacturer’s instructions to identify the 5′ ends of each gene using the gene specific primers: 5′-GGGTGGAAG-GCAAGGATTGCGGAGGAG-3′ (ELL) or 5′-TGCTGGC-TGTTGGGATCTGTAGAGGG-3′ (EAF). RACE products were cloned into pcR®2.1-TOPO®, and the inserts were sequenced to identify the 5′ end of each gene and to confirm the positions of introns. Sequences corresponding to the coding regions of the mRNAs were then subcloned into pBacPAK8 using primers overlapping consecutive exons to remove introns from *S. pombe* genomic DNA sequences.

**Strain Construction**—The *S. pombe* strain used was PP138 h− (ade6-M216 leu1–32 ura4-D18 his3-D1). *S. pombe* was grown at 32 °C in rich medium (YES) supplemented with adenine, histidine, leucine, and uracil (225 μg/ml) or minimal medium (EMM) with the supplements indicated. The *ell1Δ* strain was generated by replacing the coding region of the *ell1* gene with the kanMX6 marker as described (17).

**Analysis of Proteins Associated with FLAG-tagged SpELL and SpEAF in *S. pombe***—cDNAs encoding SpELL or SpEAF with N-terminal FLAG epitope tags were subcloned into a modified version of pNMT-TOPO (Invitrogen), which carries a thiame repressible promoter, and transformed into *S. pombe* strain PP138. Cells were grown in EMM supplemented with 10 μM thiamine and 225 μg/ml of each of adenine, histidine, and uracil. Once they had reached a density of ~5 × 10^6/ml, cells were washed in EMM supplemented with adenine, histidine, and uracil alone to release thiamine repression and grown for an additional 18 h. Cultures were harvested by centrifugation at 3,000 × g, washed in cold H₂O, and then washed in an extraction buffer containing 0.2 M Tris-HCl (pH 7.5), 0.39 M (NH₄)₂SO₄, 10 mM MgSO₄, 1 mM EDTA (pH 8.0), 20% v/v glycerol, 0.28 μg/ml leupeptin, 1.4 μg/ml pepstatin A, 0.17 mg/ml phenylmethylsulfonyl fluoride, and 0.33 mg/ml benzamidine. Cells were pulverized under liquid nitrogen by mortar and pestle, thawed, and resuspended in extraction buffer. Whole cell extracts were clarified by centrifugation at 150,000 × g before immunoprecipitation. FLAG-tagged SpELL or SpEAF and associated proteins were purified from whole cell extracts as described below under “Purification of Recombinant Proteins.”

**Expression of Recombinant Proteins in Insect Cells**—cDNAs encoding wild type SpELL and SpEAF containing N-terminal FLAG or c-Myc epitope tags, as well as an SpEAF deletion mutant lacking the first 59 amino acids, were subcloned into pBacPAK8. Recombinant baculoviruses were generated with the BacPAK expression system (Clontech). SF21 insect cells were cultured at 27 °C in SF-900 II SFM (Invitrogen). Flasks containing 1 × 10^8 SF21 cells were infected with the recombinant baculoviruses. Forty-eight hours after infection, cells were collected and lysed in 15 ml of ice-cold buffer containing 50 mM Hepes-NaOH (pH 7.9), 0.5 M NaCl, 5 mM MgCl₂, 0.2% Triton X-100, 20% (v/v) glycerol, 0.28 μg/ml leupeptin, 1.4 μg/ml pepstatin A, 0.17 mg/ml phenylmethylsulfonyl fluoride, and 0.33 mg/ml benzamidine. Lysates were centrifuged 100,000 × g for 30 min at 4 °C.

**Purification of Recombinant Proteins**—FLAG-tagged proteins were purified from SF21 cell lysates by anti-FLAG agarose immunoaffinity chromatography. Lysates from 1 × 10^8 cells were incubated with 0.5 ml anti-FLAG (M2)-agarose beads overnight at 4 °C. The beads were washed three times with Tris-buffered saline (25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2.7 mM KCl), and bound proteins were eluted from the beads with Tris-buffered saline containing 10% (v/v) glycerol and 0.3 mg/ml FLAG peptide. Where indicated, anti-FLAG-agarose eluates prepared from SF21 cells expressing both recombinant FLAG- SpELL and Myc-SpEAF were further purified by anion exchange HPLC. Eluates were adjusted to a conductivity equivalent to that of 0.05 mM KCl and applied to a 0.6-ml TSK DEAE-NPR HPLC column (Tosooh-BioSep) equilibrated in Buffer A (40 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) containing 0.1 mM KCl. The column was eluted with a 6-ml linear gradient from 0.1 to 0.5 mM KCl in Buffer A, and 0.2 ml fractions were collected. Concentrations of eluted proteins were estimated by using ImageQuant TL software (GE Healthcare) to compare the relative intensity of Coomassie Blue-stained bands corresponding to full-length proteins to the intensity of bands corresponding to BSA standards after SDS-PAGE.

**Preparation of RNA Polymerase II**—Mammalian pol II was purified from rat liver nuclei as described previously (22), except that a TSK DEAE 5-PW HPLC column was used in place of TSK DEAE-NPR.

Pol II from *S. pombe* was purified from a wild type strain (972h−) that had been grown to an OD600 of 8. The cells were dissolved at 1/2 volume/weight in 3 × lysis buffer (300 mM Tris-HCl (pH 7.9), 450 mM KCl, 3 mM EDTA, 30 μM ZnCl₂, 30 μM MgCl₂, 20% glycerol, 0.1 M β-mercaptoethanol and 0.5% Nonidet P-40) and stored at −80 °C.
DTT, 15% glycerol, and 3× protease inhibitors) and lysed using continuous flow bead beating through a Dyno-Mill (Typ KDL, Willy A. Bachofen Maschinen Fabrik, Basel, Switzerland) connected to a coolflow system from Neslab (model HX-300). The lysate was cleared by centrifugation for 45 min at 8,000 rpm in a JLA 8.1 rotor (Beckman-Coulter). The supernatant was filtered through four layers of cheesecloth and applied to a heparin column equilibrated in HSB (50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 10 μM ZnCl₂, 10 mM DTT, 5% glycerol, 1× protease inhibitors) containing 150 mM KCl. The column was washed with 3 column volumes of the same buffer and then HSB containing 600 mM KCl. The eluate was precipitated with ammonium sulfate at 50% saturation, dissolved in TEZ (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 μM ZnCl₂, 10 mM DTT, 1× protease inhibitors) until the conductivity of a 1:20 dilution was less than 100 μS/cm. The supernatant was clarified by centrifugation for 45 min at 35,000 rpm in a Ti-45 rotor and loaded onto an 8WG16 antibody-Sepharose column (CNBr-activated Sepharose coupled to monoclonal antibody 8WG16 (23) at 2 mg/ml), which had been equilibrated with TEZ containing 500 mM ammonium sulfate. The column was equilibrated to room temperature and then washed with 25 column volumes of TEZ containing 500 mM ammonium sulfate. The protein was eluted with TEZ containing 500 mM ammonium sulfate and 50% glycerol. The eluate was diluted 5-fold with TEZ and applied to a Uno-Q1 column (Bio-Rad) that was equilibrated in TEZ with 100 mM ammonium sulfate. The column was washed with 5 column volumes of the same buffer and eluted with a 30 ml linear gradient from 100 mM ammonium sulfate to 600 mM ammonium sulfate in TEZ.

Oligo(dC)-tailed Template Transcription Assays—Oligo(dC) tailed pAd-GR220 was prepared as described (24). 60-μl transcription assays were carried out in the presence of 20 mM Hepes-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 60 mM KCl, 2 mM DTT, 0.5 mg/ml BSA, 3% (w/v) polyvinyl alcohol (average molecular mass 30,000–70,000 Da), 3% (v/v) glycerol, 8 units of RNasin, 8 mM MgCl₂, ~100 ng of oligo(dC)-tailed pAd-GR220, ~25 ng of pol II, and ribonucleoside triphosphates and transcription factors as indicated in the figure legends. Reactions were preincubated for 5 min at 28 °C before the addition of ribonucleoside triphosphates. Reactions were stopped after incubation at 28 °C for the times indicated in the figures, and transcription products were resolved on 6% polyacrylamide gels containing 7 M urea, 45 mM Tris borate, and 1 mM EDTA (pH 8.3) and detected with a Molecular Dynamics Typhoon Phospholmager.

Preparation of Paused RNA Polymerase II Elongation Complexes—Paused pol II elongation complexes were assembled on oligo(dC) tailed pAd-GR220 by performing transcription reactions (scaled up 10-fold) in the presence of 50 μM ATP, 50 μM GTP, 2 μM CTP, and 10 μCi of [α-32P]CTP for 30 min at 28 °C. Free ribonucleoside triphosphates were removed by applying ~600 μl of the reaction mixture to two sequential 4-ml Sephadex G-50 spin columns equilibrated in buffer containing 20 mM Hepes-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM DTT, 0.5 mg/ml BSA, 2% (w/v) polyvinyl alcohol, 3% (v/v) glycerol, and 5 mM MgCl₂. The columns were spun for 5 min at 2,000 x g in a swinging bucket rotor, and 60 μl of eluant/reaction mixture was used in further experiments.

RESULTS

Identification of ELL and EAF Orthologs in S. pombe—ELL and EAF homologs have not been characterized in the two best studied fungal model systems, S. cerevisiae and S. pombe. To evaluate the status of these proteins in fungi and other primitive eukaryotes, we searched for orthologs of ELL and EAF using the PSI-BLAST algorithm (14). When the full-length, 621-amino acid human ELL ORF was compared with the NCBI nonredundant protein data base, the only promising candidate for a yeast ELL ortholog was an S. pombe protein encoded by the predicted SPBP23A10.14c ORF, which appeared with a borderline E-value of 0.039 after the second iteration of the search. When SPBP23A10.14c itself was used as a query in PSI-BLAST searches, it nonetheless retrieved a predicted ortholog from the sarcaromycete Yarrowia lipolytica (E-value 10⁻⁴), followed at the next iteration by predicted ELL orthologs from other fungi and higher eukaryotes (E-values between 10⁻³ and 10⁻⁸). Further iterations identified known and predicted ELL orthologs from all completely sequenced metazoa as well as from most protists and fungi but, notably, not from S. cerevisiae or from higher plants (Fig. 1A and data not shown).

The predicted SPBP23A10.14c ORF encodes a 533-amino acid protein specified by a gene with 3 exons on S. pombe chromosome II. Sequencing of a cDNA generated by 5’-RACE confirmed the predicted SPBP23A10.14c ORF. Multiple sequence alignments indicate that the sequence similarity between SPBP23A10.14c product and ELL orthologs from higher eukaryotes extends throughout the entire length of the protein, with the highest similarity concentrated in three predicted globular regions: an N-terminal all-β domain, a central region that includes a predicted all-α region, and the previously defined C-terminal occludin-like domain (Fig. 1A). We therefore designate the gene encoding the SPBP23A10.14c ORF as ell1, and we refer to the ell1 gene product as SpELL.

When the full-length 268-amino acid human EAF1 ORF was compared with sequence databases, no highly scoring fungal homologs were found; however, using the highly conserved N-terminal 110 amino acids of the human EAF1 protein as the query, we detected a low scoring match to the predicted S. pombe SPCC1223.10c ORF (E-value of 7.8 at the second iteration). The predicted SPCC1223.10c ORF in the data base is 199 amino acids long and lacks 59 N-terminal amino acids that are highly conserved in metazoan EAF proteins. Using 5’-RACE we isolated from S. pombe total RNA a longer cDNA that included a new first exon and encoded 59 N-terminal amino acids missed in the original genome annotation (Fig. 1B). When this extended protein sequence was compared with the data base, the first match, which had a highly significant E-value of 4 × 10⁻⁷, was a protein from Schistosoma japonicum (gi 30841114) annotated as similar to Homo sapiens EAF1. When this S. japonicum sequence was used as a query, the known and predicted EAF homologs from various eukaryotes were detected. These included SpEAF, which joined the rest of the family at the fourth iteration of the search with an E-value of 10⁻⁸. EAF homologs were found in all metazoa, in higher
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Yeast strains carrying mutations in genes encoding a number of proteins implicated in regulation of transcription elongation grow slowly in the presence of the nucleotide-depleting drug 6-azauracil. To determine whether ell1 or eaf1 mutants are sensitive to 6-azauracil, we generated strains lacking the ell1 or eaf1. ell1Δ and eaf1Δ strains were both viable. Although the eaf1Δ strain appeared to grow as well as wild type S. pombe on plates containing 6-azauracil, the ell1Δ strain exhibited a 6-azauracil-sensitive phenotype (Fig. 2 and data not shown).

FIGURE 1. Multiple sequence alignments of proteins from EAF and ELL families. A, distances, in amino acids, between conserved sequence blocks are indicated by numbers. Conserved regions in ELL proteins: N-terminal mostly β domain (top), α-helical domain (middle), and C-terminal occludin-like domain (bottom). Predicted secondary structure is shown below the alignment. B, N-terminal globular domains of EAF proteins. Yellow shading indicates conserved bulky hydrophobic residues (aliphatic Ile, Leu, Val or aromatic Phe, Tyr, Trp); red type indicates conserved residues with small side chains or propensity for main chain turns (Ala, Gly, Ser, Pro); red-shaded green type indicates conserved residues with carboxyl or carboxamido side chains (Asp, Glu, Asn, Gln); blue type indicates conserved positively charged residues (Lys, Arg); and magenta type indicates a conserved histidine. In the secondary structure lanes, h indicates α-helix, i indicates loop, and s indicates β-sheet. Secondary structures are predicted, except for the occludin domain structure, which is taken from Protein Data Bank structure 1XAW (C-terminal domain of human occludin) (36). Species abbreviations are as follows: Aedes, Aedes aegypti; Arath, Arabidopsis thaliana; Aspn, Aspergillus nidulans; Bos, Bos taurus; Caeb, Caenorhabditis briggsae; Chagl, Chaetomium globosum; Danre, Danio rerio; Drome, Drosophila melanogaster; Gibze, Gibberella zeae; Maggr, Magnaporthe grisea; Phano, Phaeosphaeria nodorum; Schja, Schistosoma japonicum; Schpo, Schizosaccharomyces pombe; Strpu, Strongylocentrotus purpuratus; Tetni, Tetraodon nigroviridis; Trica, Tribolium castaneum; Xenla, Xenopus laevis; Yari, Yarrowia lipolytica.

tagged SpEAF were expressed individually in S. pombe under control of the inducible nmt1 promoter. SpELL- and SpEAF-associated proteins were purified from these strains using anti-FLAG or anti-V5 agarose immunoaffinity chromatography and identified by mass spectrometry using multi-dimensional protein identification technology (MudPIT). As summarized in Table 1, epitope-tagged SpELL and SpEAF specifically copurified with endogenous SpEAF and SpELL, respectively, indicating that the two proteins interact in cells.

We next subcloned the SpELL and SpEAF ORFs into baculovirus vectors and expressed them in Sf21 insect cells in several epitope-tagged forms. In particular, we coexpressed FLAG-SpEAF with Myc-SpELL or FLAG-SpEAF with Myc-SpEAF (Fig. 3, lanes 1–4) and purified the resulting protein complexes using anti-FLAG agarose chromatography (lanes 5–12). FLAG-SpEAF copurified with Myc-SpELL (lanes 5 and 9) and FLAG-SpELL copurified with Myc-SpEAF (lanes 7 and 11). The interaction of SpELL and SpEAF appeared to be direct, since only very small amounts of any additional proteins were present in our purified preparations of SpELL/SpEAF (lanes 9 and 11). The SpELL-SpEAF Complex Activates Transcription Elongation by S. pombe RNA Polymerase II—To test the effects of SpELL and SpEAF on transcription elongation by S. pombe pol II, we assayed transcript elongation on a linearized plasmid with a single-stranded 3′-oligo(dC)-tail on its template strand. Although purified pol II is unable to initiate from a specific location on double-stranded DNA without assistance from the general transcription factors TFIIID, TFIIIB, TFIIIE, TFIIF, and TFIIH, it binds to the single-stranded oligo(dC)-tail and initiates transcription at the junction between the single- and double-stranded regions of the template (25). On the template used in our experiments, the first nontemplate strand (dT) residue is 136 nucleotides downstream of the oligo(dC)-tail; thus, transcripts of 135 nucleotides will accumulate when transcription is initiated with just ATP, CTP, and GTP. In the experiment shown in Fig. 4, transcription was initiated by the addition of purified S. pombe pol II to reaction mixtures containing the oligo(dC)-tailed template, ATP, GTP, and [α-32P]CTP. After a 30-min incubation to allow accumulation of pol II ternary elongation complexes containing radioactively labeled, 135 nucleotide long transcripts, the elongation complexes were purified by gel filtration to remove unincorporated ribonucleoside triphosphates (Fig. 4, lane 1). Nascent transcripts were then chased into longer products by the addition of ATP, GTP, CTP and UTP, in the presence or absence of SpELL, SpEAF, or both (Fig. 4, lanes 2–13). Like the mammalian ELL-EAF complex (11), the SpELL-SpEAF complex stimulated the rate of transcription elongation by its cognate S. pombe pol II, as detected by an

FIGURE 2. 6-Azauracil sensitivity of S. pombe deleted for the gene encoding SpELL. The parental strain PP138 and the ell1Δ strain, both containing the pUR19 plasmid, were grown to mid-log phase in rich media, washed in 1.2 M sorbitol, and resuspended in 1.2 M sorbitol at a density of 1 × 106 cells/ml. 5 μl of 3-fold serial dilutions of cells were spotted onto EMM plates supplemented with adenine, histidine, and leucine (225 μg/ml) with or without 6-azauracil (300 μg/ml).
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**TABLE 1**

**SpELL coimmunoprecipitates with SpEAF expressed in S. pombe**

| Peptides | Spectra | NSAF    | Peptides | Spectra | NSAF    | Peptides | Spectra | NSAF |
|----------|---------|---------|----------|---------|---------|----------|---------|------|
| SpELL    | 20      | 70      | 0.0094   | 36      | 179     | 0.0123   | 0       | 0    |
| SpEAF    | 13      | 117     | 0.0335   | 32      | 292     | 0.0425   | 0       | 0    |

**FIGURE 3. Interaction of SpELL and SpEAF.** SF21 cells coinfected with baculoviruses encoding FLAG-SpELL, Myc-SpEAF, FLAG-SpEAF, or Myc-SpEAF in the combinations indicated in the figure were prepared as described under “Experimental Procedures.” Immunoprecipitations were carried out with the antibodies indicated in the figure. Bound proteins were eluted with 150 ng/μl FLAG peptide, analyzed by SDS-PAGE, and detected by Western blotting or staining with Coomassie Blue R-250. FLAG proteins were detected with antibody anti-FLAG (M2) monoclonal antibodies and Alexa Fluor 680-labeled antimouse IgG (α light chain-specific) secondary antibodies (red). Myc-tagged proteins were detected with rabbit anti-c-Myc antibodies and IR DyeTM 800-labeled goat anti-rabbit IgG secondary antibodies (green). Fluorescently labeled secondary antibodies were detected using a Li-Cor Odyssey infrared imaging system.

increase in the rate that radioactively labeled, 135-nucleotide-long transcript was chased into longer products when reactions included the SpELL–SpEAF complex. Addition of either SpEAF or SpELL alone had a negligible effect on the rate of transcript elongation. Notably, SpEAF (60–251), which is the truncated version of SpEAF encoded by the predicted SPCC1223.10c ORF and which lacks the most highly evolutionarily conserved N-terminal region of the protein, was inactive even in the presence of SpELL, although SpEAF (60–251) could be coimmunoprecipitated with SpEAF from lysates of insect cells coinfected with viruses encoding the two proteins (Fig. 5C).

To obtain additional evidence that the SpELL–SpEAF complex possesses transcription activity, we further fractionated the purified SpELL–SpEAF complex by ion exchange HPLC and monitored copurification of SpELL–SpEAF with pol II stimulatory activity. SF21 insect cells were coinfected with baculoviruses expressing FLAG-SpELL and Mso-SpEAF, and the resulting cell lysates were subjected to anti-FLAG agarose chromatography followed by chromatography on a TSK DEAE-NPR HPLC column. FLAG-SpELL and Mso-SpEAF proteins coeluted from the TSK DEAE-NPR column at ~160 mM KCl, with the majority of both proteins being detected in fractions 7–9 (Fig. 5A, lower panel). SpELL and SpEAF purified according to this procedure were stably associated with one another, since SpELL and SpEAF eluted from the TSK DEAE-NPR at distinct positions when they were expressed and purified individually (Fig. 5A, upper panel). In contrast, heterodimers of SpELL and SpEAF (60–251) were not stable to ion exchange chromatography (data not shown), suggesting that the conserved N terminus of SpEAF contributes to the interaction between SpELL and SpEAF.

To assay column fractions for their ability to stimulate the rate of transcript elongation, we used a modified version of the tailed template assay, in which we measured accumulation of the 135-nucleotide, U-less transcript initiated from the oligo(dC)-tailed template reaction reactions were initiated by the addition of 50 μM ATP, 50 μM GTP, 2 μM CTP, and 10 μCi [α-32P]CTP (400 mCi/mmol). After a 30-min incubation, pooled pol II elongation complexes were purified, and labeled transcripts were chased into longer products by the addition of 2 μM ATP, 2 μM GTP, 2 μM CTP, and 2 μM UTP, with or without 5 pmol of SpELL or SpEAF. Reactions were stopped after 5, 10, or 30 min of chase. The lane marked M shows OX174 Haell restriction fragments used as size markers.
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The SpELL-SpEAF Complex Stimulates Pyrophosphorolysis—During RNA synthesis, RNA polymerases catalyze nucleophilic attack by the 3'-hydroxyl group of the nascent transcript on the α-phosphate of the incoming ribonucleoside triphosphate, resulting in nucleotide addition and pyrophosphate release. In the presence of high concentrations of inorganic pyrophosphate, RNA polymerases will catalyze the reverse reaction, pyrophosphorolysis, during which the nascent transcript is shortened with concurrent release of ribonucleoside triphosphates. These reactions are thought to be carried out by the same active site in pol II. If, as has been proposed for other pol II elongation factors including mammalian TFIIF, Elongin, and quantifiable alternative to the pulse-chase protocol, since it measures synthesis of a single transcript, rather than a collection of products with variable lengths. As shown in Fig. 5D, the SpELL-SpEAF complex coeluted with pol II stimulatory activity from the TSK DEAE-NPR column, arguing that the SpELL-SpEAF complex possesses intrinsic transcription activity.

The SpELL-SpEAF Complex Stimulates Pyrophosphorolysis—Previous studies have shown that metazoan ELL can bind directly to pol II in vitro (4, 7). Similarly, SpELL can bind directly to S. pombe pol II, as can SpELL-SpEAF; however, free SpEAF binds only poorly to pol II (Fig. 7A). In addition, mutations in human ELL that interfere...
FIGURE 7. Physical and functional interaction of the SpELL-SpEAF complex with pol II. A—0.2 μg of S. pombe pol II was mixed with ~10 pmol of the factors indicated, in buffer containing 20 mM Hepes-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM DTT, 0.5 mg/ml BSA, 2% (w/v) polyvinyl alcohol (average molecular mass 30,000–70,000 Da), 3% (w/v) glycerol, 5 mM MgCl₂. Immunoprecipitations were carried out with anti-FLAG-agarose, and bound proteins were eluted with 150 μg/ml FLAG peptide, analyzed by SDS-polyacrylamide gel electrophoresis, and detected by Western blotting. B, insect cells were infected with recombinant baculoviruses encoding either FLAG-HsELL, FLAG-HsEAF1, FLAG-SpELL, or FLAG-SpEAF. Proteins were then purified by anti-FLAG-agarose chromatography, and 3 pmol of each factor was assayed for its ability to stimulate transcription by pol II purified from rat liver nuclear extracts as indicated. Transcription reactions were initiated from the T-less cassette of oligo(dC)-tailed template pAd-GR220 by the addition of 50 μM ATP, 50 μM GTP, 2 μM CTP, and 10 μCi of [α-32P]CTP (400 mCi/mmol). Reactions were stopped after 5 min. C, 3 pmol of each factor was assayed for its ability to stimulate transcription by pol II purified from S. pombe. Reactions were initiated by the addition of 50 μM ATP, 50 μM GTP, and 10 μCi [α-32P]CTP (400 mCi/mmol) and stopped after 5 min.

with pol II binding also interfere with its ability to stimulate elongation (4). Taken together, these observations suggest that ELL increases the elongation activity of transcribing pol II through direct interactions with the enzyme. If this model is correct, one might expect ELL-EAF to stimulate pol II in a species-specific fashion. To determine whether the activity of SpELL and SpEAF is specific for S. pombe pol II or whether it can also stimulate mammalian pol II, we assayed the ability of purified pol II from rat liver to generate 135-nucleotide transcripts initiated from the oligo (dC)-tail on pAd-GR220, in the presence of recombinant SpELL, SpEAF, or both. As shown in Fig. 7B, transcription by mammalian pol II was unaffected by SpELL/SpEAF, although the same amount of SpELL/SpEAF stimulated elongation by S. pombe pol II. Similarly, transcription by S. pombe pol II was unaffected by recombinant human ELL/EAF1, although the same amount of human ELL/EAF1 stimulated elongation by rat pol II (Fig. 7C). Thus, stimulation of elongation by ELL/EAF depends on specific interactions between ELL/EAF and pol II.

DISCUSSION

In this report, we have applied a combination of bioinformatic and biochemical approaches to identify in S. pombe a new pol II transcription elongation factor that shares structural and functional properties with the ELL-EAF complex from higher eukaryotes. Like the mammalian ELL-EAF complex, the SpELL-SpEAF complex is capable of activating the rate of transcription elongation and promoting pyrophosphorolysis by its cognate S. pombe pol II. Genetic analysis revealed that S. pombe strains lacking the gene encoding SpELL are sensitive to the drug 6-azauracil. Mutations of yeast genes involved in a variety of cellular processes can give rise to a 6-azauracil-sensitive phenotype (30); however, this phenotype is often associated with mutations of yeast genes encoding components of the pol II transcription elongation machinery, including pol II subunits and elongation factors such as SII (also known as TFIS), Spt4, Spt5, and Spt6 (31–34). Thus, our findings are consistent with the possibility that the SpELL-SpEAF complex participates in regulation of transcription elongation in S. pombe.

Our investigation of S. pombe ELL-EAF has also provided new biochemical information about the mechanism by which ELL-EAF activates transcription elongation by pol II. First, by demonstrating that the S. pombe and human ELL-EAF complexes specifically activate S. pombe and mammalian pol II, respectively, we provide additional support for the hypothesis that they stimulate elongation through direct interaction with pol II. Second, we have shown that SpELL, like mammalian ELL, binds directly to pol II but that SpEAF binds pol II only very weakly or not at all. Thus, EAF is likely to contribute to the elongation stimulatory activity of the ELL-EAF complex by altering ELL activity, rather than by directly affecting pol II. How EAF affects ELL activity is unclear. EAF could act by altering the conformation of ELL such that it more effectively stimulates pol II elongation activity and/or binds with a greater affinity to pol II. Although the data presented in this and previous studies indicate that ELL alone is capable of binding pol II, more quantitative binding assays will be needed to determine whether EAF functions at least in part by increasing the affinity of ELL for pol II.

Importantly, our identification of the SpELL-SpEAF complex in S. pombe provides strong evidence that transcription elongation factors of this class are not limited to multicellular organisms. It is intriguing that homologs of ELL and EAF are found throughout metazoa and in a variety of fungi except S. cerevisiae and related yeasts. Notably, S. cerevisiae is distinguished from other fungi and from higher eukaryotes by its lack of several enzyme systems, including those needed for regulating many splicing processes and for RNAi (35). It is possible that the ELL-EAF complex evolved to ensure proper and timely transcription of the longer intron-containing genes in eukaryotes from S. pombe to mammals or to help coordinate post-transcriptional processing or silencing of mRNA. Because S. pombe is a genetically tractable eukaryote with more sophisticated pathways of transcriptional and post-transcriptional regulation, it should be possible to exploit S. pombe to gain new insights into the roles of the ELL-EAF complex in cells.

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