Although the PITSLRE protein kinases are members of the cyclin-dependent kinase superfamily, their cellular function is unclear. Previously we demonstrated that the general RNA splicing factor RNPS1 is a specific PITSLRE p110 kinase interactor in vivo. This suggests that the PITSLRE family of protein kinases is involved in some aspect of RNA processing or transcription. Here we identify multiple transcriptional elongation factors, including ELL2, TFIIF, TFIIS, and FACT, as PITSLRE kinase-associated proteins. We demonstrate that PITSLRE p110 protein kinases co-immunoprecipitate and/or co-purify with these elongation factors as well as with RNA polymerase II. Antibody-mediated inhibition of PITSLRE kinase specifically suppressed RNA polymerase II-dependent in vitro transcription initiated at a GC-rich (adenosine deaminase) or TATA box-dependent (Ad2ML) promoter, and this suppression was rescued by readdition of purified PITSLRE p110 kinase. Together, these data strongly suggest that PITSLRE protein kinases participate in a signaling pathway that potentially regulates or links transcription and RNA processing events.

Regulation of transcription and RNA processing occurs on many levels, and these two dynamic processes are physically linked within the cell nucleus. It is hypothesized that, in part, regulation of transcription occurs through the active exchange of associated factors with the RNAP II complex during these processes, resulting in transcriptional stimulation or repression (1–3). This hypothesis is based on the identification of numerous positive and negative regulatory transcription factors/complexes, as well as RNA processing enzymes, in association with RNAP II. Transcriptional elongation is facilitated by cellular factors that include FACT and elongator (2, 4). Complexes that repress transcription at some stage in this process include NAT (negative regulator of active transcription), sin3, and NELF (negative elongation factor) (5–7). Many of these complexes exert their effects through direct or indirect association with the RNAP II carboxy-terminal domain (CTD). The mammalian RNAP II CTD is composed of 52 heptapeptide repeats with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS) that are essential for viability (8). The CTD is heavily phosphorylated in vivo, and many protein kinases have been identified that modify this domain. In addition to regulation of transcription initiation and elongation, CTD phosphorylation has been linked to RNA processing events that result in capped, spliced, and tailed mRNA (3, 9–14). Clearly, phosphorylation plays an important role in regulating the production of mRNA transcripts by affecting the nature of the RNAP II enzyme complex.

Several cdks phosphorylate the CTD, and some of these cdks co-purify with RNAP II complexes. These kinases include cdk1, cdk7, cdk8, and cdk9. It is likely that sequential phosphorylation events, as well as phosphorylation of specific residues by specific protein kinases, help regulate transcription from preinitiation through termination. For example, a characterization of cdk7 and cdk8 demonstrated biochemically distinct CTD kinase activity (15). Cyclin C/cdk8 interacts with RNAP IIa (hypophosphorylated CTD) complexes that are competent for initiation of transcription (16). Cyclin H/cdk7/MAT1 (menage à trois 1) is part of the general transcription factor IIF (TFIIH) and interacts with initiation and early elongation complexes (17–19). A recent report demonstrates that phosphorylation of cyclin H by cyclin C/cdk8 represses the ability of TFIIH to activate transcription and to phosphorylate the CTD (20). Cyclin T and cdk9 (PITALRE) were identified as components of the positive transcription elongation factor, which is active after initiation and is responsible for a CTD kinase activity that promotes transcript elongation (21–23).

Additional cdk family members exist with unknown functions, including the PITSLRE and the PCTAIRE protein kinases (24). Two distinct but closely linked human PITSLRE kinase genes (Cdc2L1 and Cdc2L2) express at least 20 PITSLRE protein kinase isoforms (25). PITSLRE kinase homologues have been identified in numerous organisms, including chicken, mouse, Drosophila melanogaster, Caenorhabditis elegans, and Schizosaccharomyces pombe (Refs. 26–28; GenBankTM CAA18412.1). The highly conserved nature of these protein kinases and the data accumulated to date suggest...
important roles in both cell proliferation and apoptosis. The amino-terminal halves of the PITSLRE p110 isoforms contain several distinct domains, including a RD/RE (single amino acid codons for Arg, Asp, and Glu) motif and a highly acidic region of glutamic acids. An RNA-binding protein (29) and general activator of pre-mRNA splicing (30), RNPS1, associates with the amino-terminal domain of p110 in vivo. PITSLRE p110 is localized in the nucleoplasm as well as in nuclear speckles (i.e. intrachromatin granule clusters (IGCs)). IGCs are unique sub-nuclear structures that appear to be storage and assembly sites for RNA processing and transcription factors, including RNAP II (31, 32). The recruitment of many RNA processing factors from IGCs to active sites of transcription, which are often located at the periphery of the IGCs and in the nucleoplasm, is regulated by phosphorylation/dephosphorylation (33, 34). These data suggest that the PITSLRE p110 isoforms may regulate some aspects of RNA synthesis and processing.

Based upon our findings that PITSLRE p110 kinases interact with the general splicing factor RNPS1, as well as with several transcriptional elongation factors reported herein, we believe that PITSLRE kinases may play a role in the regulation of transcription and/or transcript processing. This hypothesis is further supported by experiments demonstrating that the PITSLRE p110 kinases co-purify with complexes containing RNAP II and co-immunoprecipitate with RNAP II in vivo. The transcriptional elongation factor ELL2 was isolated in a two-hybrid interactive screen using only the carboxyl-terminal catalytic domain of the PITSLRE p110 protein kinase and was subsequently shown to associate with PITSLRE p110 in vivo. TFIIF and TFIIS, additional transcriptional elongation factors, associate with PITSLRE p110 in mammalian cells by co-immunoprecipitation after extensive purification of transcriptional- RNA processing complexes from the nucleus. Furthermore, both subunits of FACT, an elongation factor that facilitates the transition through nucleosomes, were identified as components of the PITSLRE affinity purified complexes by electrospray capillary liquid chromatography/tandem mass spectrometry (LC-MS-MS) analysis. We also show that PITSLRE is present in transcriptionally active complexes. Finally, introduction of both affinity-purified PITSLRE monoclonal and polyclonal antibodies, which recognize the carboxy-terminal protein kinase domain, into in vitro transcription assays specifically suppressed transcript synthesis irrespective of whether the transcription initiated from a TATA-dependent or TATA-independent promoter. This suppression was alleviated by the addition of purified PITSLRE p110 protein back into these reactions. Furthermore, antibodies generated to the amino-terminal domain, which excludes the protein kinase domain, had no effect. Taken together, these results strongly support our hypothesis that PITSLRE p110 kinases may play some role in the production of translatable RNA transcripts in proliferating cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—All cell lines were obtained from the American Type Culture Collection and cultured as specified. Transfections were carried out using either LipofectAMINE (Invitrogen) or FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Antibodies—Polyclonal antibodies to RNPS1 were prepared by immunizing rabbits with a recombinant GST-RNPS1 fusion protein encoding the amino-terminal half of the protein (RNPS1-Δ1, amino acids 1–136), which was produced in bacteria (Rockland). Antibodies were affinity-purified sequentially by first running the serum over GST-coupled Sepharose 4B and then running the flow-through over GST-RNPS1-Δ1 coupled to Sepharose 4B. The antibodies were released with a pH shock (100 mM glycine, pH 2.5) and neutralized with 1 M Tris, pH 8.8. The PITSLRE antibodies P2N100 and GN1 have been described previously (29, 35). PITSLRE mouse monoclonal antibody P1C was produced by immunizing mice with the P1C antigen encoding the last 75 amino acids of PITSLRE p110 as described previously (36).

Commercial antibodies used include TFIIF RAP 74 (C-18), TFIHH p62 (Q-19), Pol II (N-20), edk8 and FLAG polyclonal antibodies from Sigma Chemical Co.; and Anti-C-Myc, Anti-HA, and Anti-Flag M2 monoclonal antibodies from Clontech Laboratories; cyclin C polyclonal antibody from Neo-Marker, Inc.; and anti-FLAG M2 affinity gel from Eastman Kodak Company. A highly purified Mono S column fraction containing the purified RNAP II and its associated protein subunits, prepared by investigators in the laboratory of Dr. D. Reinberg according to previous protocols, was used for the two immunoprecipitations shown in the experiment shown in Fig. 5B (37). Immunoblotting was performed as described previously (29).

Recombinant Protein Production and in Vitro Binding Assays—GST-RNPS1-Δ1 and -Δ2 were described previously (29). The full-length ELL2 cDNA was cloned into pGEX 4T for the production of GST-ELL2. The TFIIS cDNA was obtained by polymerase chain reaction, sequenced, and cloned into pGEX 2T for production of GST-TFIIS and into pCDNA3.1 with an amino-terminal FLAG tag for expression in mammalian cells.

For the in vitro association assay between GST-ELL2 or GST-RNPS1-Δ2, and PITSLRE p110, CEM-C7 cells were washed in phosphate-buffered saline and resuspended in 50 mM Hepes, pH 7.9, 150 mM NaCl, 15 mM MgCl2, 0.1 mM EDTA, 1% Nonidet P-40, 0.1% Triton X-100, 0.2 M NaF, and 10 mM β-glycerophosphate, and 1× complete protease inhibitors (Roche Molecular Biochemicals). The lysate was incubated on ice for 10 min, sonicated for 10 s, and centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4 °C. The supernatant was preclariﬁed by rotation with GST-Sepharose beads for 1 h at 4 °C and a low speed centrifugation. The cleared lysate was then diluted 1.5× into Pol II buffer (10 mM Hepes, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 0.1 mM Na2VO3, 0.2 M NaF, 10 mM β-glycerophosphate, and 1× complete protease inhibitors). GST-ELL2 or GST-RNPS1-Δ2 beads plus lysate were incubated rotating for 2 h at 4 °C, and the resulting bead complexes were washed four times in 1 ml of Pol II buffer, boiled in protein sample buffer, and analyzed by SDS-PAGE and immunoblot.

Immunoprecipitation—The cells were lysed in Pol II buffer as described above for experiments described in Figs. 3, 4B, and 5A. For commercial antibodies, 5 μg of antibody was used per mg of lysate. All immunoprecipitations were washed four times in 1 ml of Pol II buffer or the same buffer containing 150 mM NaCl, unless otherwise noted. For Fig. 4B, the cell lysate was preclariﬁed with protein A-agarose and normal mouse serum before immunoprecipitation overnight using the monoclonal antibody M2, the His monoclonal antibody plus protein A-agarose, or the P2N100 polyclonal antibody plus protein A-agarose in Pol II lysis buffer. The immunoprecipitations were washed four times with Pol II buffer in the presence of 50–150 mM NaCl. In Fig. 5A, lysate was combined with antibody and Gammabind Plus Sepharose (Amersham Biosciences, Inc.) and then rotated for 2 h at 4 °C. In Fig. 5B the incubation was performed overnight for part A and 2 h for part B.

Chromatography—A cellulose phosphate P11 (Whatman) cation exchange column was loaded with 650 μg of HeLa nuclear extract protein (prepared as described in Ref. 38) and washed with P11 buffer (20 mM Hepes, pH 7.9, 0.1 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The proteins were eluted by step gradient using 0.1, 0.3, 0.5, 0.8, and 1.0 M KCl P11 buffer. The 0.1 M KCl P11 complexes were subjected to mass spectrometry analysis. The 0.1 M KCl P11 complexes were dialyzed overnight against 0.1 M KCl-P11 buffer and then loaded onto DEAE-anion exchange column (Bio-Rad). The proteins were step-eluted with 0.1, 0.3, 0.5, 0.8, and 1.0 M of KCl in column buffer (20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). For further biochemical purification of PITSLRE p110 complex, the DEAE 0.5 M fractions containing PITSLRE p110 were loaded onto a SP-Sepharose column (Amersham Biosciences, Inc.) and then a Mono S column (Amersham Biosciences, Inc.). The Mono S fractions containing PITSLRE p110 were then loaded onto an UNO-Q (Bio-Rad) column, and the proteins were step-eluted with 0.1, 0.3, 0.5, 0.8, and 1.0 M of KCl in column buffer (20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). For further biochemical purification of PITSLRE p110 complex, the DEAE 0.5 M fractions containing PITSLRE p110 were further purified by an affinity chromatography column containing P2N100 polyclonal antibody covalently linked to Sepharose. The resulting affinity purified and biochemically purified PITSLRE p110 complexes were subjected to mass spectrometry analysis.
and 65 kDa were used to calibrate the column. Jurkat JT-1 cells were washed in phosphate-buffered saline and lysed in 20 mM Tris, pH 7.9, 100 mM KCl, 0.1% Nonidet P-40, 0.2 mM EDTA, 10 mM β-mercaptoethanol, and 1× complete protease inhibitors. The lysate was incubated on ice for 10 min, sonicated for 10 s, and centrifuged twice at 14,000 rpm in a microcentrifuge for 10 min. 1 ml of lysate (0.1 ml) was loaded onto the Superose 6 column, and 1-ml fractions were collected. Immunoblot analyses using 30 µl of each fraction were performed.

**Mass Spectrometry Analysis**—The protein samples were digested with endoproteinase Lys-C and trypsin according to the method of Link et al. (39). The resulting peptide mixture was analyzed by combined capillary liquid chromatography/tandem mass spectrometry. Mass spectrometry was performed using a Thermoquest LCQ-DECA ion trap mass spectrometer with an electrospray ion source. Product ion (MS2) spectra were subjected to search using the SEQUEST program of Eng and Yates (ThermoQuest).

In Vitro Transcription Assays—In vitro transcription reactions were carried out as described previously (38) with minor modifications. The supercoiled template DNA was purified by double banding in cesium chloride centrifuged gradients. Transcription reaction mixtures (25 µl of total volume) containing 8 mM Hepes, pH 7.9, 40 mM KCl, 6 mM MgCl₂, 0.08 mM EDTA, 0.2 mM dithiothreitol, 0.2 mM dithiothreitol, 8% glycerol, 30 units of RNase T1, 100 mM of template DNA, 9–10 µM of HeLa nuclear extract (Promega), and different amounts of affinity-purified antibodies and purified recombinant protein were incubated at 30 °C for 10 min prior to the addition to final concentrations of 0.2 mM of ATP, CTP, GTP, and UTP. 8 nM UTP, 0.05 mM 3′-O-methyl-guanosine 5′-triphosphate (Amersham Biosciences, Inc.), and 1 µM [α-32P]UTP (80 Ci/mmol). The reaction mixtures were then incubated for 1 h at 30 °C and stopped by the addition of 175 µl of stop solution (0.3 mM Tris-HCl, pH 7.4, 0.3 mM sodium acetate, 0.5% SDS, 2 mM EDTA, 25 µg/ml RNA) containing an end-labeled extraction control DNA fragment. The reaction solutions were extracted twice with phenol/chloroform/octanol alcohol (25:54:1, v/v/v), and nucleic acids in the reaction were precipitated by the addition of 600 µl of ethanol and incubation at −70 °C for 30 min. Following centrifugation at 13,000 × g for 10 min at 4 °C, the pellet was air-dried and dissolved in 4 µl of nuclelease-free water. Equal volume of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue) was added to the in vitro transcribed RNA. These RNA solutions were then heated at 90 °C for 10 min and electrophoresed in a 6% denaturing (7 µurea) polyacrylamide gel containing 0.5× TBE buffer (22.5 mM Tris-borate and 0.5 µM Ethylenediaminetetraacetic acid). The amount of radiolabeled transcript and extraction control bands were then quantitated using a PhosphorImager (Molecular Dynamics).

Gal4-binding Domain Construct and Two-hybrid Screening—The DNA sequence encoding the catalytic domain of the PITSLRE A protein kinase derived from a caspase 3 cleavage (40) was cloned in-frame with the Gal4 DNA-binding domain of the pAS2 vector (kindly provided by Dr. S. Elledge, Baylor College of Medicine). A human B-cell cDNA library subcloned into the pACT plasmid containing the Gal4 DNA-binding domain of the pAS2 vector (kindly provided by Dr. S. Elledge, Baylor College of Medicine) was screened with an endoproteinase Lys-C and trypsin according to the method of Link et al. (41). The carboxyl-terminal domain of PITSLRE p110 is localized within the cytoplasm (data not shown) and therefore cannot associate with RNAP II or other transcription factors.

**RESULTS**

**PITSLRE p110 Isoforms Co-elute with Large Molecular Mass Complexes**—Jurkat total cell lysate was fractionated over a Superose 6 column by fast protein liquid chromatography and then analyzed by immunoblotting using the PITSLRE p110-specific antibody P2N100 and the RNAP II large subunit antibodies 8WG16 and N20. In the lane labeled Jurkat cell lysate, 30 µg of unfractionated cell lysate was used. All other lanes represent 30 µl from each fractionated lysate. B, similar Superose column size fractionation of Jurkat cell lysates transfected with the PITSLRE N370 construct (containing only the amino-terminal domain; Ref. 41) was performed. The carboxy-terminal protein kinase domain of PITSLRE p110 is localized within the cytoplasm (data not shown) and therefore cannot associate with RNAP II or other transcription factors.

**FIG. 1. PITSLRE p110 kinase isoforms fractionate with large molecular mass complexes.** A, Jurkat cell lysates were fractionated over a Superose 6 column by fast protein liquid chromatography and then analyzed by immunoblotting using the PITSLRE p110-specific antibody P2N100 and the RNAP II large subunit antibodies 8WG16 and N20. In the lane labeled Jurkat cell lysate, 30 µg of unfractionated cell lysate was used. All other lanes represent 30 µl from each fractionated lysate. B, similar Superose column size fractionation of Jurkat cell lysates transfected with the PITSLRE N370 construct (containing only the amino-terminal domain; Ref. 41) was performed. The carboxy-terminal protein kinase domain of PITSLRE p110 is localized within the cytoplasm (data not shown) and therefore cannot associate with RNAP II or other transcription factors.

co-fractionation with RNAP II, the same experiment was performed with a truncated protein encoding only the amino-terminal 370 amino acids of the kinase (i.e. PITSLRE N370; Ref. 40). The majority of the PITSLRE N370 protein is associated with the fractions containing the >1-MDa protein complexes, which also contain RNAP II (Fig. 1B). Thus, the amino-terminal 370 amino acids of PITSLRE p110 appear to be sufficient for PITSLRE p110 association in large molecular mass RNAP II complexes. Examination of RNPS1 distribution by size exclusion chromatography resulted in a very similar distribution to large molecular mass complexes as seen for PITSLRE p110 and RNAP II (data not shown). Thus, two distinct large molecular mass complexes (i.e. >1-MDa and ~700 kDa) containing RNAP II also include a significant portion of the PITSLRE p110 protein kinase.

PITSLRE p110 isoforms associate with transcription complexes containing both hypophosphorylated and hyperphosphorylated forms of the RNAP II large subunit. Biochemical purification of PITSLRE p110-containing complexes was undertaken to characterize the proteins contained in the large molecular mass complexes described above. Soluble HeLa cell nuclear extract was fractionated over a series of columns as outlined in Fig. 2. The final eluants were analyzed to identify proteins contained therein. RNAP II was identified by electrospray LC-MS-MS as a PITSLRE co-purifying protein. To further characterize the association of PITSLRE p110 kinase with the RNAP II large subunit, CEM C7 cell lysates were subjected to immunoprecipitation with either PITSLRE P2N100 or RNAP II 8WG16 antibodies. The resulting PITSLRE antibody-immunoprecipitated complexes were analyzed by immunoblot for the presence of the RNAP II large subunit using the 8WG16 and H5 monoclonal antibodies. The 8WG16 monoclonal antibody specifically recognizes the unphosphorylated form of the repeat within the CTD, whereas the H5 monoclonal antibody is specific for phosphorylated serine at position 2 within the conserved CTD repeat (YSPTPSP) of the RNAP II large subunit. The results shown in Fig. 3A indicate that both the hyperphosphorylated (~240 kDa) and hypophosphorylated (~220 kDa) forms of the RNAP II large subunit co-immunoprecipitated with PITSLRE p110. Immunoblotting of a membrane identical to the leftmost panel of Fig. 3A with an affinity-purified form of

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the RNAP II large subunit. The panel indicates the hypophosphorylated and hyperphosphorylated forms of the RNAP II larger subunit, respectively.

Experimental Procedures. The untransfected cells and hypophosphorylated forms of the RNAP II larger subunit, respectively. The antibodies used to detect RNAP II include the polyclonal N20, which recognizes the first 20 amino acids of the largest subunit of RNAP II, and the monoclonal H14 antibody, which specifically recognizes phosphorylated serine at position 5 within the consensus CTD repeat (YSPTSPS). Fig. 3B shows, like Fig. 3A, that both the hypophosphorylated and hyperphosphorylated forms of RNAP II are associated with PITSLRE p110.

Identification of ELL2 as a PITSLRE p110 Kinase in Vivo Interactor—Yeast two-hybrid interactive screens were performed to identify proteins that specifically interact with the PITSLRE protein kinase catalytic domain. The carboxyl-terminal half of the PITSLRE p110 polypeptide, which encodes the kinase catalytic domain and a short, unique carboxyl-terminal domain of unknown function, was cloned in-frame with the Gal4 DNA-binding domain of the pAS2 vector. A human B-cell cDNA library subcloned into the pACT plasmid containing the Gal4 activation domain was transformed into a Y190 yeast strain containing the PITSLRE bait, and the entire screening process was carried out as described previously (29). A positive clone was obtained, and its cDNA insert was sequenced in its entirety on both strands. The corresponding sequence was then analyzed using the Genetics Computer Group programs and the GenBank^TM data base (Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children’s Research Hospital). The open reading frame lacked a methionine at the 5’ end, and thus the nucleotide sequence was used to search the expressed sequence tag data base. Overlapping expressed sequence tag clones were identified and sequenced, and the full-length open reading frame was assembled using a convenient restriction enzyme site shared by both fragments. The full-length clone was identified as ELL2, the sequence of which was published at the same time we completed the sequence analysis of this PITSLRE carboxyl-terminal interactive cDNA (42).

To determine whether full-length ELL2 also interacted with the PITSLRE p110 isoforms, bacterially expressed and glutathione-Sepharose bead-purified GST-ELL2 and GST-RNPS1-D2 fusion proteins were incubated with CEM C7 cell lysates. The resulting complexes were washed and subjected to immunoblot analysis for PITSLRE p110. RNPS1, a positive control for these experiments, has previously been shown to interact with the amino-terminal domain of the PITSLRE p110 protein kinase in two-hybrid screens and in vivo (29). The results of this experiment indicated that, similar to GST-RNPS1, the GST-ELL2 protein associates with PITSLRE in vitro (Fig. 4A). GST beads lacking the ELL2 sequence did not bring down the PITSLRE p110 kinase.

To examine the in vivo interaction of ELL2 with PITSLRE p110, NIH 3T3 cells were transfected with a FLAG-ELL2 construct. Cell lysate from transfected cells was immunoprecipitated with the antibody indicated above each lane according to the "Experimental Procedures." The CEM C7 lanes are total cell lysate alone. The labels RNAP II and IIA represent hyperphosphorylated and hypophosphorylated forms of the RNAP II large subunit, respectively. B, HeLa cells were transfected with either PITSLRE p110-FLAG or FLAG-RNPS1 constructs. Lysates from the transfected cells were immunoprecipitated with M2 anti-FLAG-agarose, and the immunoprecipitated fractions were subjected to immunoblot analysis for RNAP II using the antibody indicated below each panel. The arrows to the right of each panel indicate the hypophosphorylated and hyperphosphorylated forms of the RNAP II large subunit. The HeLa lanes represent cell lysate from untransfected cells.

The data in Fig. 3A indicate that PITSLRE p110 is associated with a portion of both the hypophosphorylated (IIA) and hyperphosphorylated (IIO) forms of RNAP II. To confirm this result, FLAG epitope-tagged versions of PITSLRE p110 and RNPS1 were transiently expressed in HeLa cells. The FLAG-tagged proteins were immunoprecipitated from the cell lysates with M2 beads, and the immunoprecipitated complexes were analyzed for the presence of the RNAP II large subunit by immunoblot. The antibodies used to detect RNAP II include the polyclonal N20, which recognizes the first 20 amino acids of the largest subunit of RNAP II, and the monoclonal H14 antibody, which specifically recognizes phosphorylated serine at position 5 within the consensus CTD repeat (YSPTSPS). Fig. 3B shows, like Fig. 3A, that both the hypophosphorylated and hyperphosphorylated forms of RNAP II are associated with PITSLRE p110.

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NIH 3T3/ELL2 lanes and to immunoblot analysis using the PITSLRE P2N100 antibody.

The CEM C7 lane represents total cell lysate. His8-p110 (6His-p110) represents a purified full-length PITSLRE p110 isoform as a positive control. B, NIH 3T3 cells were transfected with ELL2-pFlex and pKerRSV in a 20:1 molar ratio. After 2 days, the cells were split as needed and maintained on G418 selection (200 μg/ml) and 5% fetal calf serum for 4 weeks. Lyse from ~107 cells was divided and immunoprecipitated with the antibodies indicated above the lanes as described under “Experimental Procedures.” Immunoprecipitates were subjected to immunoblot analysis using the PITSLRE P2N100 antibody. CEM C7 and NIH 3T3/ELL2 lanes represent lysate. His6-p110 (6His-p110) was purified from insect cells and used as a positive control. The interaction of ELL2 with PITSLRE p110 was maintained following wash conditions including up to 150 mM NaCl and 0.5% Nonidet P-40.

of up to 150 mM NaCl and 0.5% Nonidet P-40. However, we have noted that the ELL2 and PITSLRE p110 interaction is more easily detected following immunoprecipitation in a buffer containing 50 mM salt as compared with buffers containing 100–150 mM salt. In vitro immunoprecipitation kinase assays indicated that ELL2 was not a substrate of the PITSLRE p110 protein kinase.

PITSLRE kinases associate with general transcription elongation factors TFIIH and TFIIA in vivo. Because the PITSLRE p110 protein kinase, RNAPII, and ELL2 were identified as likely components of the larger molecular mass PITSLRE p110 protein complexes, a possible physical association between PITSLRE p110 and other components of the RNAPII elongation complex was examined in more detail by co-immunoprecipitation/mass spectrometry studies. Sonicated HeLa cell lysates were immunoprecipitated with antibodies against PITSLRE p110, the RNAPII amino terminus (N20), RNPS1, TFIIH (p62 subunit), or TFIIH (RAP 74 subunit), respectively. The resulting immune complexes were analyzed by immunoblot for the presence of the PITSLRE p110 kinase. In fact, RNAPII, RNPS1, and TFIIH, but not TFIIH, co-immunoprecipitated with the PITSLRE p110 protein kinase (Fig. 5A). In addition, each of the immunoprecipitated complexes was examined by immunoblot for the presence of the RNAPII large subunit, RNPS1, cyclin H/cdk7, and RAP 74. As expected, each of these immune complexes contained all of the proteins for which the immunoprecipitation was performed (data not shown). Furthermore, the TFIIF subunits Rap30 and Rap74 also co-purify with PITSLRE p110 complexes as determined by immunoblot (Fig. 2).

GST-TFIIS beads were used in an in vitro binding assay as described above to examine possible association with PITSLRE p110 because an appropriate human TFIIS antibody was not readily available. This experiment indicated that TFIIS and PITSLRE p110 are also associated (Fig. 5A). In addition, endogenous PITSLRE p110 protein kinase co-immunoprecipitated with transiently expressed FLAG-tagged TFIIS from mammalian cells (data not shown). Thus, PITSLRE p110 is found in association with multiple transcriptional elongation factors both in vitro and in vivo. However, PITSLRE p110 did not co-immunoprecipitate with similarly transiently expressed epitope-tagged ELL, elongin A, or elongin B proteins. These results strongly suggest that the PITSLRE p110 kinase associates specifically with those RNAPII complexes containing a subset of elongation factors, including ELL2, TFIIF, and TFIIS.

Co-purification of FACT with PITSLRE-containing Complexes—FACT is an enhancer of transcriptional elongation composed of two subunits, hSPT16 and SSRP1 (4). Both of these FACT subunits were identified by immunoblot, as well as by LC-MS-MS, following each of the PITSLRE p110 complex purification schemes illustrated in Fig. 2. This is not entirely unexpected because FACT also participates in certain aspects of the transcriptional elongation process. However, at this time we do not know whether the co-purification of FACT with the PITSLRE p110 protein kinase is relevant to the regulation of FACT activity or its associations with other proteins. Additional studies are underway that will hopefully establish any potential functional link between FACT and the PITSLRE p110 protein kinase.

PITSLRE p110 Is Present in Active Transcription Complexes—To further demonstrate that the PITSLRE p110 kinases and RNAPII are found in the same large molecular mass complexes in vivo, a highly purified, transcriptionally active Mono S column fraction containing human RNAPII complexes (37) was examined using two different approaches. The first method involved direct immunoblot analysis of this purified Mono S column fraction using the specific PITSLRE p110 kinase affinity-purified polyclonal antibody P2N100. The second method involved immunoprecipitation of TFIIH and its associated proteins using an appropriate antibody, followed by immunoblot analysis for the PITSLRE p110 protein kinase (Fig. 5B). This Mono S fraction has previously been shown to contain, among other factors, TFIIE, TFIIF, TFIIH, cdk8, and cyclin C (Ref. 37 and data not shown). Both of the methods described above demonstrated that the PITSLRE p110 protein kinase was present in the purified RNAPII complexes contained in this Mono S fraction. Moreover, the signal obtained for PITSLRE p110 following TFIIH immunoprecipitation (50 μl of column fraction) was roughly equivalent to that of the Mono S fraction alone (10 μl of column fraction), suggesting that 15−20% of the PITSLRE p110 from this fraction is found in complexes containing TFIIH. PITSLRE p110 detection was judged to be specific, because immunoprecipitation of this fraction using a lactate dehydrogenase antibody (as a negative control) did not bring down this protein kinase.

Inhibition of PITSLRE Activity Suppresses Transcription in Vitro—To determine whether the observed associations between the PITSLRE p110 kinase and the various transcriptional elongation factors described above is functionally significant, we examined whether the PITSLRE p110 kinase is required for transcription using a standard in vitro assay. The
amount of transcript produced from both an Ad2ML expression construct (controlled by a conventional TATA-like promoter) and an adenosine deaminase gene expression construct (controlled by a GC-rich promoter) was significantly reduced following the addition of either the affinity-purified PITSLRE P1C monoclonal (Fig. 6) or polyclonal antibodies to the transcription reaction mixture. Conversely, affinity-purified antibodies directed against the very amino-terminal (P2N100) and central (GN1) domains of PITSLRE p110 were also tested, and there was no reduction in transcriptional activity. It is important to note that the PITSLRE P1C monoclonal and polyclonal antibodies bind the catalytic domain of PITSLRE p110, and preliminary data indicate that these antibodies inhibit its protein kinase activity, as judged by a decrease in the ability of the kinase to undergo autophosphorylation. Using 1.4 μg of PITSLRE P1C antibody, transcription from both the TATA (e.g. Ad2MLP) and GC (e.g. adenosine deaminase) promoters was reduced to background levels observed from a promoter-less construct (PC2AT). Thus, the protein kinase activity of PITSLRE p110 may help regulate some aspect of the transcription process.

When insect cell purified His₆-PITSLRE p110 protein kinase was added to the reactions containing 1.4 μg of P1C monoclonal antibody, transcription returned to 70% of the control level (Fig. 6). Both the wild-type and kinase-inactive forms of PITSLRE p110 protein kinase were equally effective in recovery of transcriptional activity, suggesting that titration of the P1C antibody by either form of the protein was responsible for the recovery of transcriptional activity. The observed PITSLRE inhibition-induced suppression of transcript production is consistent with the interpretation that the association between the PITSLRE p110 protein kinase and the various transcriptional elongation proteins is functionally relevant. Moreover, the data suggest that transcriptional activity is specifically being suppressed through the binding of antibodies corresponding to the PITSLRE p110 carboxyl-terminal kinase domain. Further study of PITSLRE p110 complexes (i.e. the >1-MDa and 700-kDa fractions, as well as those associated with the nuclear matrix) will most likely reveal the details of the mechanism responsible for the observed effects of the PITSLRE p110 protein kinase on transcription.

FIG. 5. PITSLRE p110 kinase isoforms interact with TFIIF and TFIIIS transcriptional elongation factors in vitro and in vivo and are present in an active transcription complex. A, HeLa lysates (0.5 mg/lane) were immunoprecipitated (IP) with the antibodies indicated above each lane. The resulting membrane was then immunoblotted with the PITSLRE P2N100 antibody as described under “Experimental Procedures.” The one exception is the TFIIS lane, which represents an in vitro binding assay (see “Experimental Procedures”). The HeLa lane represents total cell lysate. B, Mono S-purified RNAP II fractions (50 μl immunoprecipitation, 10 μl in lane containing Mono S fraction only) were immunoprecipitated with the antibodies indicated above each lane. The resulting proteins were then immunoblotted using the PITSLRE P2N100 antibody.

DISCUSSION

The identification of the transcriptional elongation factor ELL2 as a PITSLRE p110 interactor through two-hybrid interactive screening analysis, as well as the in vivo demonstration of an association between this protein kinase and several additional transcriptional elongation factors, has revealed that the PITSLRE p110 protein kinase may play a role in transcriptional elongation. Because PITSLRE p110 and cdk9, a component of positive transcription elongation factor that promotes transcription (43), are related members of the cdk family, this may not be necessarily unexpected. However, unlike positive transcription elongation factor/cdk9, PITSLRE p110 kinases are associated with large multi-protein complexes that contain RNAP II, at least four distinct transcriptional elongation factors (i.e. ELL2, TFIIF, TFIIIS, and FACT), and an RNP protein that functions as a general activator of RNA splicing (i.e. RNPS1). Furthermore, it is somewhat intriguing that within the large cohort of cdc2-related protein kinases, at least four members (i.e. cdk7, cdk8, cdk9, and now PITSLRE) have been associated with regulating certain aspects of transcription and RNA processing within the cell.

Transcriptional elongation factors can be grouped into three classes (44). The first class includes those factors that prevent RNAP II arrest (TFIIS). The second class includes those factors that prevent transient pausing of RNAP II (ELL, ELL2, and TFIIF). The third class includes those factors that promote transcriptional elongation by modifying chromatin structure (FACT). It is interesting that the PITSLRE p110 protein kinase associates with transcriptional elongation factors of all three classes. Although ELL2, TFIIF, TFIIIS, and RNAP II do not appear to be substrates of the PITSLRE p110 protein kinase, their function may be regulated by its kinase activity in an indirect manner (e.g. phosphorylation of an inhibitory/stimulatory factor). Purified His₆-tagged PITSLRE p110 kinase derived from insect cells had no discernable effect when added to in vitro tailed template RNAP II elongation assays (data not shown). It should be noted, however, that this highly purified, minimal component assay is not very sensitive to exogenously added protein kinase activity, such as TFIIF. Additionally, it is also possible that a cyclin-like regulatory partner is necessary for PITSLRE p110 kinase activity, and in its absence no direct effects of the PITSLRE p110 kinase on transcriptional elongation can be observed using this minimal component assay. This study establishes that transcription complexes containing the
PITSLRE p110 protein kinase and elongation factors exist in vivo, and it also implicates this protein kinase in some aspect of the regulation of transcription/RNA processing.

An alternative, but not mutually exclusive, hypothesis is that the PITSLRE p110 protein kinase acts as a bridge between proteins in these elongation and RNA processing complexes and that it regulates either their association or their activity, thereby linking transcription and splicing. Intriguingly, attempts to establish stable human cell lines expressing the amino-terminal domain or the entire PITSLRE p110 protein kinase have been unsuccessful. In contrast, stable expression of the PITSLRE p46 protein kinase catalytic domain has been attained without difficulty (40). Furthermore, expression of the PITSLRE amino-terminal protein region (amino acids 1–370) is indicated by arrows – 370) is PITSLRE amino-terminal protein region (amino acids 1–370) is present in the proper assembly and/or function of transcription/RNA processing complexes.

We have now demonstrated that the PITSLRE p110 protein kinase is found in large molecular mass multi-protein complexes that potentially contain a general RNA splicing factor (i.e. RNPS1; Ref. 29), transcriptional elongation factors, and the RNAPII large subunit. Moreover, PITSLRE p110 and RNPS1 associate with complexes containing the hyperphosphorylated form of RNAPII, a form of the protein associated with transcriptional elongation events and stimulation of pre-mRNA splicing (14). Finally, interference with PITSLRE p110 protein kinase activity by antibody binding significantly and similarly reduces transcript production from either a TATA-dependent or GC-rich (and TATA-independent) promoter template. These observations, taken as a whole, indicate that at least a portion of soluble nuclear RNAPII complexes contain the PITSLRE p110 protein kinase and strongly suggest that this kinase may regulate some aspect of transcription and/or RNA processing. We are currently working to further purify PITSLRE p110 protein kinase-containing complexes from the nucleoplasm, IGCs, and the transcriptionally active regions associated with the nuclear matrix to identify additional, unknown interacting proteins (e.g. a cyclin-like regulatory protein) as well as a potential substrate(s).

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