We report the cDNA cloning and functional characterization of human cyclin L, a novel cyclin related to the C-type cyclins that are involved in regulation of RNA polymerase II (pol II) transcription. Cyclin L also contains a COOH-terminal dipeptide repeat of alternating arginines and serines, a hallmark of the SR family of splicing factors. We show that recombinant cyclin L interacts with p110 PITSLRE kinase, and that cyclin L antibody co-immunoprecipitates a kinase activity from HeLa nuclear extracts that phosphorylates the carboxyl-terminal domain (CTD) of pol II and splicing factor SC35, and is inhibited by the cdk inhibitor p21. Cyclin L antibody inhibits the second step of RNA splicing in vitro, and recombinant cyclin L protein stimulates splicing under suboptimal conditions. Significantly, the IC50 for splicing inhibition by p21 is similar to the IC50 for inhibition of the cyclin L-associated kinase activity. Cyclin L and its associated kinase are thus new members of the pre-mRNA processing machinery.

Cyclins and their partners the cyclin-dependent kinases (cdks)1 reviewed in Refs. 1 and 2) may be classified into two major groups according to their function: the cell cycle regulators, which include the cyclin classes A, B, D, and E and cdks 1, 2, 3, 4, and the transcriptional regulators, comprising the cyclin classes C, H, K, and T and cdks 7, 8, and 9. These latter cyclin/kinase pairs are associated with the transcriptional machinery, and are components of transcription factor TFIIH (3–7), elongation factor P-TEFb, (8–10), and the RNA polymerase II holoenzyme (11–13). Human cyclin K is homologous to cyclin C, associates with the large subunit of pol II, and its kinase partner, cdk9, phosphorylates the CTD of pol II (14).

Phosphorylation of the CTD plays a pivotal role in regulating transcription initiation, elongation, and processing of RNA transcripts. It is widely accepted that transcription and RNA processing are linked (reviewed in Ref. 15): capping enzymes, polyadenylation factors, and splicing factors assemble at the CTD, and these interactions are modulated by CTD phosphorylation (reviewed in Refs. 16–18). Extensive research has focused on the role of the CTD in regulating pre-mRNA splicing. The CTD targets splicing factors to transcription sites in vitro (19); phosphorylated pol II stimulates splicing (20), and splicing factors associate with pol II through a hyperphosphorylated CTD (21).

Splicing factors comprise the small nuclear ribonucleoprotein particles, the spliceosome-associated proteins, and the SR proteins (reviewed in Ref. 22). SR proteins constitute a conserved family of pre-mRNA splicing factors that are characterized by an arginine-serine dipeptide repeat within their carboxyl-terminal domain and one or two RNA-binding domains within their amino-terminal domain (23). SR proteins are essential splicing factors, and are capable of complementing splicing-deficient cellular extracts. Several members of this family have been identified, and among these the human factors ASF/SF2 (24, 25) and SC35 (26) are well characterized. Four additional SR proteins with molecular masses of 30, 40, 55, and 75 kDa have also been identified (27). SR proteins are required for early steps in spliceosome assembly and influence selection of splice sites (22, 26, 28). In contrast, the SR-related proteins contain an RS repeat but lack RNA-binding domains (reviewed in Ref. 29). A recent survey of the human, yeast, Drosophila melanogaster, and Caenorhabditis elegans genomes has identified a number of other RS domain proteins. These include proteins involved in 3′-end processing, chromatin-associated proteins, kinases, phosphatases, and a new cyclin, named cyclin L, that was identified in C. elegans and D. melanogaster (30), and it is similar to cyclin ania-6a in the mouse (31). Cyclin L is related to the transcriptional cyclin K (14), and it is the first known example of a cyclin containing an RS domain in addition to a cyclin box. The function of cyclin L is not known although results obtained from immunofluorescence and immunoprecipitation experiments show that the mouse homologue, cyclin ania-6a, localizes to nuclear speckle compartments, associates with the hyperphosphorylated form of RNA pol II, the splicing factor SC-35, and the cdk PITSLRE (31). These results suggest a potential role for cyclin L in RNA splicing.

We report here the cloning and characterization of the human gene for cyclin L, and show that recombinant human cyclin L interacts with p110 PITSLRE kinase. Moreover, cyclin L is associated with a kinase activity that phosphorylates histone H1, the CTD, and SR protein SC35. This activity is inhibited by low concentrations of the cdk-specific inhibitor p21. An antibody to cyclin L inhibits in vitro splicing specifically at the second step, and recombinant cyclin L protein stimulates splicing of a β-globin precursor RNA. Furthermore, in vitro splicing is inhibited by p21 with an inhibition profile nearly identical to that of kinase inhibition. These results provide initial evidence that cyclin L is a functional cyclin and directly affects pre-mRNA splicing, although the precise mechanism remains to be elucidated.

EXPERIMENTAL PROCEDURES

Molecular Cloning of Human Cyclin L cDNA—Human cyclin L cDNA was generated by PCR using primers designed to match the 5′- and 3′-ends of a contiguous sequence of expressed sequence tags that appeared to encode a novel cyclin. The primers were: sense, 5′-CAGTCTTTG-
TTCCGGTGTTCCGGTCGTT-3' and antisense, 5'-AAACAAAGATTTGATTTTATTTCTCCGT-3'. These primers were used to amplify the cDNA clone from human lung cDNA (CLONTECH) using a thermostable polymerase mixture (Advantage cDNA polymerase, CLONTECH). The PCR product was excised from a low-melting point agarose gel, digested with XbaI, and cloned into the T-A vector pCR-II-TOPO (Invitrogen, The Netherlands), and sequenced in both directions using an Applied Biosystems 373A sequencer.

**Northern Blots—**A membrane containing 2 μg of poly(A)+ RNA from eight different human tissues (CLONTECH) was probed with a 738-bp PCR fragment spanning positions 111–849 of the cyclin L cDNA. 20 ng of the PCR fragment was labeled using the High Prime DNA Labeling Kit (Roche Molecular Biochemicals) and purified using a Chromaspin-30 column (CLONTECH). The hybridization, stripping, and re-hybridization procedures were as described by the manufacturer, except that the last wash was carried out at 42 °C.

**GST Fusion Protein Expression—**The coding sequence of the cyclin L cDNA clone described above was amplified using primers containing an overlapping EcoRI recognition site (upstream primer, 5'-GGTGCTGAATTTCCAGGATTTTAATTTTATTTCTCCGT-3' and downstream primer, 5'-GTGGCAGATCCGGCCGCTTGTGCTGCATTGTAGCTC-3'). The PCR product was purified, digested with EcoRI, and cloned into the EcoRI site of pGex2T (Amersham Biosciences). A plasmid containing the correct orientation of the insert as confirmed by sequencing was used for protein expression. Cultures of freshly transformed XL1 Blue cells harboring pGex2T (Amersham Biosciences) were grown to an A600 of 1.0, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, incubated for an additional 2 hours, and harvested by centrifugation. Cells were lysed for 45 min in ice-cold TBS (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 2 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.5 μg/ml pepstatin) and lysozyme (0.2 mg/ml). The crude lysate was sonicated, and Triton X-100 was added to a final concentration of 1% (v/v). Cell debris was removed by centrifugation at 12,000 × g for 30 min. The supernatant was incubated for 30 min at 4 °C with washed glutathione-Sepharose beads, followed by three washes with phosphate-buffered saline, and eluted in 10 mM glutathione in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% phenylmethylsulfonyl fluoride. Proteins were dialyzed in phosphate-buffered saline + 0.1% Nonidet P-40. This extensive washing was necessary to reduce nonspecific binding. The washed beads were either used directly for kinase assays, or bound proteins were eluted in SDS sample buffer (at 4 °C) with 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and incubated for 1 h in antibody/Blotto solution. After washing three times for 10 min in TBST, the filter was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Bio-Rad) (1:2,000). After extensive washing of the membranes, signals were detected using the SuperSignal West Pico chemiluminescent substrate (Pierce).

**Immunoprecipitation and Kinase Assays—**Protein A-Sepharose was washed three times in A100 buffer (20 mM Hepes-OH, pH 7.2, 100 mM KCl, 0.1 mM EDTA, 0.2 mM dithiothreitol) and incubated overnight at 4 °C with 10 μl of bovine serum albumin (1.5 mg/ml) and 10 μl of antibody (as indicated). The complex was washed as before, and aliquots of the purified complex were incubated with 80 μg of HeLa nuclear extract for 1 h at 4 °C. The completed complexes were washed as before with A100 + 0.1% Nonidet P-40 followed by three washes with A100. The pellets were resuspended in A100 to give a total volume of 120 μl. 60 μl each were added to 10 μl of a kinase reaction containing 20 μM ATP, 1 μl of [γ-32P]ATP (150 μCi/μl), 6 mM MgCl2, and the kinase substrate as indicated. The reaction was incubated at ambient temperature for 1 h, precipitated with 25% (v/v) trichloroacetic acid, washed with acetone, and air-dried. Pellets were resuspended in SDS-loading buffer, boiled, and separated by SDS-PAGE. Gels were dried and exposed to Bio-Max film or quantified on a PhosphorImager (Molecular Dynamics).

**In Vitro Splicing Assays—**The precursor RNA used in _in vitro_ splicing assays was transcribed from plasmid pSP64H80 containing a β-globin 3' splice site. The transcription reaction mixture consisted of RNA polymerase (Promega), the labeled RNA was run on a 6% denaturing polyacrylamide gel, the gel was briefly exposed to film, and the band corresponding to the full-length RNA was cut from the gel. RNA was eluted from the gel slice in elution buffer (0.3 M Na-acetate, pH 5.3, 33% buffer-saturated phenol, 60 μg of glycerol) for 3 h at ambient temperature or overnight at 4 °C. The sample was centrifuged, and the supernatant was purified through a Ultrafree CL filter (Millipore), aliquoted, precipitated separately with ethanol, and stored as dry pellets at −50 °C. Pellets were resuspended in RNase-free H2O at an estimated concentration of 6 μg/ml (or 40 fmol/μl) and 1–2 μl were used in _in vitro_ splicing reactions. Splicing reactions were performed as described (33) and contained 80 mM potassium acetate, 4 mM magnesium acetate, 20 mM creatine phosphate, 1 mM freshly prepared ATP, 1 unit/ml RNasin (Promega), labeled β-globin RNA precursor, 40 μg of HeLa nuclear extract (Promega), or 20 μg HeLa nuclear extract plus 200 ng of GST-cyclin L fusion protein, in a total volume of 20 μl. Reactions were incubated at 30 °C for 3 h (or as indicated), stopped with 150 μl of stop buffer (0.5% SDS, 0.3 M Na-acetate, pH 5.3, in TE) plus 20 μg of glycerol, extracted with phenol/chloroform and precipitated with ethanol. The reaction products were run on 6% denaturing polyacrylamide gels with a kinase-labeled 100-bp ladder (PerkinElmer Life Sciences), and were visualized by exposure to Bio-Max film and phosphorimage analysis.

**RESULTS**

**Cloning of a Novel Cyclin with Homology to the SR Protein Family of mRNA Splicing Factors—**A 2076-bp human cyclin L cDNA was cloned by PCR from a human lung cDNA library (GenBank™ accession number AF180920). The first methionine codon, at nucleotides 55–57, is in a strong sequence context for an initiation consensus sequence (54), suggesting that this is the correct initiation codon. The ORF encodes a theoretical 59.6-kDa protein, 526 amino acid residues in length, with an isoelectric point of 10.71. A classical polyadenylation signal indicated. The reaction was incubated at ambient temperature for 1 h, precipitated with 25% (v/v) trichloroacetic acid, washed with acetone, and air-dried. Pellets were resuspended in SDS-loading buffer, boiled, and separated by SDS-PAGE. Gels were dried and exposed to Bio-Max film or quantified on a PhosphorImager (Molecular Dynamics).

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Cyclin L contains a cyclin box, an RS repeat, and is expressed in multiple tissues. A, alignment of cyclin L (L) (accession number AAD53184) with the cyclin box of cyclin K (K) (accession number AAF82290). Similar residues are indicated by a plus sign (+). B, alignment of cyclin L with the RS repeat of splicing factor ASF/SF2 (accession number AAD53184) with the cyclin box of cyclin K (accession number AY034790). Both the RS repeats and the cyclin boxes are shown as boxes (not to scale). The unfilled portions of the boxes represent the 5'- and 3'-untranslated regions. The sizes of the exons and introns are shown in base pairs. The position of the CpG island is shown. The initiation and 3' in-frame stop codons are indicated. C, alternatively spliced variants of cyclin L. Schematic representation of the exon structures of three human cyclin L transcripts. The 14 exons are shown as boxes (approximately to scale).

The Human Cyclin L Gene—The human cyclin L gene is located on chromosome 3 in the 3q23.2–3 region. The cyclin L gene is 12.4 kb in length, and is encoded by 14 exons (Fig. 2A). The gene is generated by read-through of the donor splice sites from exons 5 to the polyadenylation signal. A cDNA for cyclin L was obtained by screening a human lymphocyte cDNA library. Consistent with this finding, peripheral blood lymphocytes express a large amount of the 4.5-kb cyclin L mRNA (Fig. 1C).

Characterization of Anti-cyclin L Antibody—In an effort to clone the human homologue for the B' subunit of the yeast transcription factor TFIIIB, we used a rabbit polyclonal antibody raised against the yeast protein (kindly provided by Drs. A. TATA-less promoter is encompassed by the 5'-flanking region of the cyclin L gene. There is a CpG island (67% GC) that encompasses the gene and the equivalent of exon 8 is absent from the mouse gene. Alternatively Spliced Transcripts of Human Cyclin L mRNA—cDNA cloning revealed that the human cyclin L gene generates a number of alternatively spliced mRNA transcripts. The major transcript has been designated the α transcript that encodes a 526-amino acid residue ORF (Fig. 2B). Exons 4, 7, and 8 are skipped in cyclin L mRNA. Cyclin Lα mRNA has 92% identity with the mouse cyclin ania-6a ORF (GenBank™ accession number AF159159) (31). The only region of divergence between human cyclin Lα and mouse ania-6α is close to the NH2 terminus in a repetitive region. Two other human transcripts encode truncated proteins. The β transcript skips exons 4, terminates in exon 7, and encodes a 232-amino acid residue ORF (GenBank™ accession number AF367476). The γ transcript encodes an 172-amino acid residue ORF, includes exon 4, and terminates within this exon (GenBank™ accession number AF0934790). Both the β and γ transcripts are conserved in mouse, where it was shown that the truncated variant is not targeted to the nucleus and does not associate with RNA pol II and splicing factors (31).

As noted above, there are 2 major cyclin L transcripts of 2.3 and 4.5 kb detected on a Northern blot (Fig. 1C). The 2.3-kb band likely represents the α and β transcripts, and an analysis of human and mouse expressed sequence tag data suggests that the 4.5-kb band may represent the γ transcript. Cyclin Lγ is generated by read-through of the donor splice sites from exons 5 to the polyadenylation signal. A cDNA for cyclin Lγ was obtained by screening a lymphocyte cDNA library. Consistent with this finding, peripheral blood lymphocytes express a large amount of the 4.5-kb cyclin Lγ mRNA (Fig. 1C).
A Cyclin-dependent Kinase Activity Is Associated with Cyclin L—Anti-L antibody was used to immunoprecipitate cyclin L and any potential kinase partner(s) from a HeLa nuclear extract. Immunoprecipitates were bound to Protein A-Sepharose, washed extensively, and used in an in vitro kinase assay. The substrate specificity of the cyclin L-associated kinase was analyzed with recombinant RNA pol II CTD (GST-CTD), histone H1, splicing factors SC35 and SRp46, GST-cyclin L fusion protein as test substrates, and bovine serum albumin as a negative control. The cyclin L-associated kinase specifically phosphorylates the CTD of pol II, histone H1, and SC35 (Fig. 4A). In contrast, SRp46, GST-cyclin L, and bovine serum albumin are not substrates for the cyclin L-associated kinase. The lack of phosphorylation of cyclin L and SRp46 demonstrates that the cyclin L-associated kinase is not a general SR protein kinase. As an additional control, these same substrates were tested with immunoprecipitates formed with preimmune serum, and as expected, no kinase activity was detected (Fig. 4A, lanes labeled PI).

To confirm that the kinase activity was associated with cyclin L, and was not an artifact of the immunoprecipitation procedure, we incubated purified GST-cyclin L protein with HeLa extract, captured GST-cyclin L, and associated proteins on glutathione-Sepharose beads and used the washed beads in an in vitro kinase assay. A potent kinase associates with GST-cyclin L, but not with GST alone as evidenced by the phosphorylation of histone H1 (Fig. 4B). In additional experiments, we compared the kinase activity of immunoprecipitates formed with anti-cyclin L with those isolated with other antibodies. We used anti-TFIIBA as negative control, and antibodies to the p62 subunit of TFIIB for cdk7 activity, and anti-ckk8 as positive controls for CTD phosphorylation (Fig. 4C). The CTD kinase activity of the anti-cyclin L IP is comparable with that of anti-p62 and anti-ckk8. Anti-cyclin L and anti-p62 IPs also phosphorylate histone H1, whereas anti-ckk8 immunoprecipitates failed to phosphorylate H1 as expected (11). No signifi-
cant levels of kinase activity were detected with the preimmune serum and no antibody control reactions.

To provide an independent confirmation that a kinase activity is associated with cyclin L, we partially purified cyclin L and associated proteins from HeLa nuclear extracts through three successive rounds of ion exchange chromatography. Fractions containing cyclin L were identified by immunoblotting with anti-cyclin L antibody. Following phosphoelute analysis, DEAE-Sepharose, and Mono-Q FPLC, fractions that contained cyclin L protein also exhibited potent kinase activity toward histone H1 and the CTD, with the peak of kinase activity closely corresponding to the peak of cyclin L protein (data not shown). We thus conclude that a kinase activity co-purifies with cyclin L.

Since the orphan kinase PITSLRE was identified as the cdk partner for cyclin ania-6a, the mouse homologue of cyclin L (31), we tested whether human PITSLRE associates with human cyclin L. Recombinant cyclin L-GST fusion protein, or GST alone, was incubated with the HeLa nuclear extract and then captured interacting proteins on glutathione-Sepharose beads. After extensive washing, the bound proteins were eluted from the Sepharose beads and analyzed by SDS-PAGE and Western blotting with antibody to PITSLRE (Fig. 5A). This assay relies on the exchange of proteins that interact with endogenous cyclin L with the added recombinant protein. Under our assay conditions, an excess of GST-cyclin L binds ~10% of the input PITSLRE protein, whereas no PITSLRE is bound by GST alone. These results suggest that human PITSLRE may be the cdk partner for human cyclin L, however, future experiments will address whether cyclin L interacts with other cdks and/or other nuclear proteins. We next compared the kinase activities of HeLa nuclear extract immunoprecipitates formed with antibody to cyclin L, antibody to recombinant cyclin L, and anti-PITSLRE. We find comparable kinase activities toward the CTD and histone H1 with each of these antibodies (not shown). We next examined whether the cyclin L-associated kinase activity is inhibited by known cdk inhibitors, such as p21, that selectively inhibits G1/S cyclin-cdk complexes (37). Kinase assays with anti-cyclin L immunoprecipitates and GST-CTD, SC35, and histone H1 as test substrates were performed in the absence or presence of increasing concentrations of p21. We also tested the effect of p21 on anti-PITSLRE and anti-rL immunoprecipitates. Fig. 5B shows that the cyclin L-associated kinase is effectively inhibited by p21, with an estimated IC50 of 5–10 nM, and that the inhibition profile is identical for the immunoprecipitates with anti-L, anti-rL, and anti-PITSLRE (Fig. 5C). Taken together, our results strongly suggest that p110 PITSLRE is the functional kinase partner for human cyclin L.

Anti-cyclin L Antibody Inhibits Splicing—We next determined whether cyclin L has a direct effect on in vitro RNA splicing. Previously, a monoclonal antibody recognizing the RS repeat in several SR proteins specifically inhibited in vitro splicing of a β-globin precursor RNA that confirmed the functional role for RS repeats in pre-mRNA splicing (38). Although anti-L is a polyclonal antibody, and the epitopes are unknown, we nevertheless tested its effect on in vitro splicing of a β-globin precursor RNA, as an initial attempt to determine a functional role for cyclin L in splicing. Labeled RNA precursor was incubated with HeLa nuclear extract under splicing conditions for 2 h at 30 °C in the absence or presence of increasing concentrations of rabbit preimmune serum, anti-cyclin L serum, or a monoclonal antibody that recognizes SR proteins (mAB1H4) as a positive control. RNA was purified and analyzed by denaturing PAGE. Anti-cyclin L inhibits splicing activity (Fig. 6A, lanes 6–10) similar to anti-SR (lanes 11–15), whereas preimmune serum has no effect (lanes 1–5). This result strongly suggests that cyclin L is involved in splicing of pre-mRNAs, although the pattern of inhibition differs between anti-cyclin L and mAB1H4 antibodies. For anti-cyclin L, the signals corresponding to the finished products (exon 1 fused to exon 2, intron lariat and linear intron, indicated by asterisks) are significantly reduced compared with the control (lane 6) even at the lowest anti-cyclin L concentration (lane 7), and disappear with higher antibody concentrations (lanes 9 and 10). The intron-exon 2 and exon 1 intermediates (indicated by a dot) show an increase at the lowest anti-cyclin L concentration tested (lane 7), and are detectable even at high anti-cyclin L concentrations (lanes 8–10). In contrast, addition of mAB1H4 to the splicing reaction results in an immediate decrease of intermediates at the lowest antibody concentration, whereas products decrease more slowly than with anti-cyclin L. These results indicate that anti-cyclin L antibody inhibits the second

Fig. 5. Cyclin L forms an active kinase complex with p110 PITSLRE that is inhibited by the cdk-specific inhibitor p21. A, Western blot of cyclin L-GST bound proteins probed with antibody to p110 PITSLRE. 10 μL of HeLa nuclear extract was incubated with 4 μg of GST-cyclin L or GST (4 μg) in a total volume of 100 μL for 1 h at 30 °C, and bound proteins were captured with glutathione-Sepharose beads (20 μL of packed beads). After extensive washing of the beads, GST-cyclin L and GST-bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE and Western blotting with antibody to PITSLRE. As a positive control, an aliquot of the HeLa nuclear extract protein (40% of input) was analyzed. B, quantitation of a p21 titration experiment with SC35 and the pol II CTD as substrates and anti-L immunoprecipitates. Immunoprecipitates from HeLa nuclear extract were incubated with SC35 or GST-CTD in the presence of increasing concentrations of p21 and treated as described. An autoradiograph of an experiment with SC35 as substrate is shown in the inset of the graph. C, p21 titration experiment with histone H1 as substrate and anti-L, anti-rL, and anti-PITSLRE immunoprecipitates.
step of splicing, in contrast to mAB1H4, that inhibits splicing at an early stage.

As expected, the addition of antibodies recognizing various proteins that are known not to play a role in pre-mRNA splicing, did not affect splicing efficiency. In contrast to anti-L, polyclonal antibodies raised against retinoblastoma protein, cAMP-response element-binding protein, the NH2 terminus of RNA pol II, the p89 subunit of transcription factor TFIIH, and the kinase cdk6 did not inhibit splicing activity (Fig. 6B, lanes 4–13). Furthermore, anti-cyclin C did not affect splicing (not shown). These data suggest that the inhibition of splicing observed with anti-cyclin L reflects a role for this cyclin, and possibly its kinase partner, in pre-mRNA splicing.

To further investigate the specific inhibition of the second step of splicing by anti-L antibody, we performed a time course of the splicing reaction. Labeled β-globin precursor RNA was incubated with HeLa nuclear extract in the absence of antibody (lanes 1–5) or in the presence of 100 μg of anti-cyclin L (lanes 6–10) for the indicated time periods. B and C, graphic representation of a time course experiment comparing the effect of anti-cyclin L antibody (+α-L) and 1H4 (+α-SR) on splicing. Intermediates (sum of intron-exon 2 + exon 1) are shown in B, and splicing products (sum of mRNA + intron) are shown in C. The control is without added antibody (no AB). Relative splicing activity represents the percentage of the signals normalized to the total amount of input in each lane. (Note the accumulation of intermediates and the greater reduction of spliced products in the presence of anti-L compared with anti-SR.)

Recombinant GST-Cyclin L Stimulates In Vitro Splicing—Splicing experiments were performed as before except that lower concentrations of HeLa nuclear extract were used to provide suboptimal splicing conditions (20, 39, 40). Under these conditions, splicing activity was significantly reduced (Fig. 8A,
Cyclin L Is Involved in Pre-mRNA Splicing

FIG. 8. Recombinant GST-cyclin L fusion protein stimulates in vitro splicing. GST-cyclin L fusion protein was added to splicing reactions with β-globin precursor RNA and suboptimal concentrations of HeLa nuclear extract. A, the HeLa extract concentration needed to reduce to yield the splicing products was determined empirically (lane 2, compared with lane 1). Addition of GST-cyclin L protein to the splicing reaction under suboptimal conditions results in an increase of splicing products and a decrease in intermediates compared with the control (Fig. 8A, lane 3). A time course experiment allowed for a more detailed analysis of this effect. Splicing was allowed to proceed for different time intervals in the absence of added protein, or in the presence of GST or GST-L fusion protein (Fig. 8B). In the absence of added protein under suboptimal conditions, a low level of splicing intermediates is detected after 60–120 min, final products are almost undetectable under these conditions (lanes 2 and 3). The same result is obtained when recombinant GST is present in the reaction (lanes 4–6). In contrast, addition of recombinant GST-cyclin L results in a decrease in intermediates and a significant increase in final products, consistent with a potential role of cyclin L in the second step of the splicing reaction (lanes 7–9). These experiments were repeated at least three times and gave identical results. Quantitation of a time course experiment shows that GST-cyclin L enhances splicing activity ~7-fold after a 3-h incubation, compared with the control, whereas addition of GST alone is without effect (Fig. 8C).

To determine whether cyclin L contributes to the splicing reaction via its associated kinase activity, we tested splicing in the absence or presence of increasing concentrations of the cdk inhibitor p21. Significantly, splicing activity is inhibited by p21 with an IC_{50} comparable with the IC_{50} determined for the cyclin L-associated kinase activity. The inhibition curves for the relative splicing activity or relative CTD kinase activity in response to p21 are nearly superimposable (Fig. 8D). This result strongly suggests that a kinase similar or identical to the cyclin L-associated kinase PITSLRE plays an important role in in vitro splicing.

DISCUSSION

Here we describe the cloning and characterization of the gene for human cyclin L. Cyclin L is a novel protein containing an NH_{2}-terminal cyclin box and a COOH-terminal RS repeat, and it is homologous to the mouse cyclin ania-6a that was shown to localize to nuclear speckles, and associate with RNA polymerase II and the splicing factor SC35 (31). We demonstrate here that cyclin L protein is a functional cyclin, associated with specific kinase activity, and a functional protein involved in splicing. The cyclin L cyclin box is most similar to cyclin K, a member of the C-type cyclins that are thought to be involved in regulation of RNA pol II transcription (10). C-type cyclins and their kinase partners are defined by their ability to phosphorylate the CTD of pol II, and we show that cyclin L immunoprecipitates and recombinant GST-cyclin L associate with a kinase that phosphorylates the CTD in vitro. It is very likely that the CTD is a target of the cyclin L/kinase in vitro, since it was shown by immunostaining and immunoprecipita-

2 compared with the control lane 1). Addition of 200 ng of GST-cyclin L (lane 3) restored splicing activity to a level comparable with the control (lane 1). B, time course experiment under suboptimal conditions in the absence of added protein (lanes 2 and 3), in the presence of 200 ng of GST (lanes 4–6) or 200 ng of GST-cyclin L (lanes 7–9). C, quantitation of a time course experiment under suboptimal conditions in the absence of added protein (open bars), in the presence of 200 ng of GST (densely stippled bars) or 200 ng of GST-cyclin L (lightly stippled bars). The bar graph represents the sum of the splicing products (indicated by asterisks) normalized to the total input in each lane. D, inhibition of in vitro splicing with the cdk inhibitor p21. β-Globin precursor RNA was incubated with the HeLa nuclear extract in the absence or presence of increasing concentrations of p21. The relative splicing activity represents the sum of products and intermediates measured at each p21 concentration relative to the products and intermediates determined in the absence of inhibitor.
tion experiments that cyclin ania-6a interacts with the hyperphosphorylated form of RNA pol II (31). The SR protein SC35 is specifically phosphorylated in vitro by the cyclin L-associated kinase, whereas the SR protein SRp46 or recombinant cyclin L are not substrates for phosphorylation in vitro. These findings indicate that the cyclin L-associated kinase is not a general RS domain kinase, but probably phosphorylates one or several sites outside of the RS domain in specific proteins such as SC35. It is not surprising that the cyclin L-associated kinase does not phosphorylate RS repeats. This protein motif is a target for specific, non-cdk kinases of the SRPK and Ctk/Sty families. Phosphorylation of RS domains is required for their function (reviewed in Ref. 29), whereas phosphorylation of SR proteins outside of the RS domain has not been reported to date. Although it remains to be established whether SC35 is a target in vivo, our results together with the finding that cyclin ania-6a co-localizes and co-immunoprecipitates with SC35 (31) indicate that SC35 is an in vivo substrate.

The cdh PITSLRE p110 was identified as a partner for cyclin ania-6a in mice (31), and it is likely that one of the isoforms of PITSLRE is also associated with human cyclin L. This is corroborated by the finding that human p110 PITSLRE can be captured from the HeLa nuclear extract with recombinant cyclin L in a GST interaction experiment (Fig. 5A). Additionally, p21 inhibits the kinase activity of anti-PITSLRE immunoprecipitates with the same IC$_{50}$ as for anti-L and anti-rL immunoprecipitates (Fig. 5C).

CTD phosphorylation is required for transcriptional elongation and RNA processing (reviewed in Ref. 15). Therefore, cyclin H/cdk7, cyclin C/cdk8, and cyclin K/cdk9 promote transcriptional elongation and affect RNA processing indirectly through phosphorylation of the CTD. Although cyclin L is similar to the C-type cyclins, the presence of an RS domain that has been considered a diagnostic for a protein involved in splicing (22) suggests a direct involvement in pre-mRNA splicing. Furthermore, the cyclin L-kinase complex specifically phosphorylates the SR protein SC35, in addition to the CTD, and this activity is most likely found in vivo since cyclin L associates with SC35 and the CTD in vivo. It is conceivable that cyclin L and its kinase link CTD phosphorylation and RNA splicing by recruiting specific splicing factors like SC35 to the CTD. Since cyclin L is expressed at different levels in different tissues, it is also possible that it contributes to tissue-specific regulation of splicing. Differential expression patterns have been found for many other RS domain proteins and this is thought to contribute to the respective activities of different proteins in regulating splicing (22).

RS domains have been identified in a large number of proteins, most of which are involved in RNA splicing, 3’ end RNA processing, or RNA transport (reviewed in Refs. 23, 29, 41, and 42). As in cyclin L, RS domains always occur together with one or several other, unrelated domains within the same protein, and the nature and organization of these additional domains has led to classification of RS domain proteins into several groups. SR proteins, represented by prototypical ASF/SF2 and SC35, contain a COOH-terminal RS domain and one or two NH$_2$-terminal RNA recognition motifs, and are essential splicing factors necessary for early spliceosome assembly and regulation of constitutive and alternative splicing. SR-related proteins are splicing regulators, and can contain RNA recognition motifs, CTD-interacting domains, U2AF-binding domains, or a DEEX/DH box, a motif found in RNA-dependent ATPases/putative helicases. The SR protein-specific kinase Ctk/Sty contains a kinase domain in addition to an RS repeat. The modular organization of cyclin L is typical for an RS domain protein, however, cyclin L is the first known example of an RS domain protein containing a cyclin box.

The highest degree of homology of the RS domains in cyclin L with that of splicing factor ASF/SF2 resides within a stretch of 42 amino acid residues that are 77% similar and 66% identical. The entire RS domain in cyclin L however, extends from residues 383 to 519, a region of 136 residues that is highly enriched in arginine and serine residues, most of which occur as pairs and together account for 51% of the amino acid residues in this region. A similar long RS domain is found in splicing factors SC35 and 9G8 (26, 43), in contrast to ASF/SF2 and SRp20 that contain RS domains of 35 and 49 residues, respectively. It has been suggested that differences in RS domains reflect differences in protein-protein interactions and may partly account for specificity by which various RS domain proteins select their partners.

We showed that an antibody recognizing cyclin L inhibited in vitro splicing. This result indicates that the epitope(s) in cyclin L, recognized by anti-L, are functional elements in the splicing reaction. Moreover, the inhibition specifically affects the second step of the reaction, in contrast to the inhibition observed with the monoclonal antibody mAB11H4 (or 11H4G7) that inhibits formation of splicing intermediates. Another monoclonal antibody recognizing RS repeats in SR proteins, mAB16H3, was previously shown to inhibit pre-mRNA splicing in vitro at an early step (38), consistent with a role for SR proteins in early steps in spliceosome assembly. Although it cannot be ruled out that the inhibition by anti-L is because of steric hindrance, the finding that recombinant GST-cyclin L stimulates splicing specifically at the second step strongly supports the notion of a potential involvement of cyclin L in the second step of splicing. Moreover, the finding that p21 also inhibits splicing and the cyclin L-associated kinase activity with similar IC$_{50}$ values, strongly suggests that the cyclin L-associated kinase is involved in splicing. Although the precise function of cyclin L in the splicing reaction remains to be elucidated, our results suggest a role for the cyclin L-kinase complex in the second step of splicing.

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