Dim1p Is Required for Efficient Splicing and Export of mRNA Encoding Lid1p, a Component of the Fission Yeast Anaphase-Promoting Complex

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Schizosaccharomyces pombe Dim1p is required for maintaining the steady-state level of the anaphase-promoting complex or cyclosome (APC/C) component Lid1p and thus for maintaining the steady-state level and activity of the APC/C. To gain further insight into Dim1p function, we have investigated the mechanism whereby Dim1p influences Lid1p levels. We show that S. pombe cells lacking Dim1p or Saccharomyces cerevisiae cells lacking its ortholog, Dib1p, are defective in generalized pre-mRNA splicing in vivo, a result consistent with the identification of Dim1p as a component of the purified yeast U4/U6.U5 tri-snRNP complex. Moreover, we find that Dim1p is part of a complex with the splicing factor Prp1p. However, although Dim1p is required for efficient splicing of lid1+p pre-mRNA, circumventing the necessity for this particular function of Dim1p is insufficient for restoring normal Lid1p levels. Finally, we provide evidence that Dim1p also participates in the nuclear export of lid1+p mRNA and that it is likely the combined loss of both of these two Dim1p functions which compromises Lid1p levels in the absence of proper Dim1p function. These data indicate that a mechanism acting at the level of mRNA impacts the functioning of the APC/C, a critical complex in controlling mitotic progression.

The fission yeast Schizosaccharomyces pombe provides an excellent model organism for the analysis of cell cycle regulation. In particular, genes involved in the G2/M transition and in progression through mitosis have been identified and studied extensively. Entry into mitosis depends upon Cdc2p function, the single Cdk in fission yeast. Cdc2p activity depends both upon its association with Cdc13p, a B-type cyclin, and upon the balance between positive and negative regulatory phosphorylation events (22). Beyond its activation, however, our understanding of how Cdc2p promotes the events of mitosis is limited.

In an effort to identify downstream targets of Cdc2p function which coordinate entry into mitosis, we had isolated second-site mutations, one of which was dim1-35, capable of reducing the restrictive temperature of a novel cdc2 mutant, cdc2-D217N (5, 6). When shifted to restrictive temperature, dim1-35 mutant cells proceed through mitosis in the absence of nuclear division, demonstrating an uncoupling of proper DNA segregation from other cell cycle events. In contrast, deletion of dim1 from the S. pombe genome produces a lethal G2 arrest. Lethality is rescued by overexpression of the mouse dim1+p homolog, mdim1. Deletion of the Saccharomyces cerevisiae dim1 homolog, DIB1, is also lethal. Both mdim1 and dim1+p are capable of rescuing lethality of the dib1::HIS3 mutant. Interestingly, dim1-35 cells display sensitivity to the microtubule-destabilizing drug thiabendazole. In the presence of this drug, dim1-35 cells proceed through mitosis and display a cut (cell untimely torn) phenotype. dim1-35 cells also lose minichromosomes at elevated rates (5). These properties led us to suggest that Dim1p was involved somehow in the entry and transit of S. pombe cells through mitosis.

Dim1p is a highly conserved, essential 17-kDa protein (5). Although structurally it is a member of the thioredoxin superfamily (31, 42), the catalytic sites present in thioredoxin are absent in Dim1p, and the biochemical function of Dim1p has yet to be elucidated. In an effort to further understand dim1+p function, a synthetic lethal screen was performed with the temperature-sensitive dim1-35 mutant, and lid (lethal in dim1-35) mutants were isolated. In a tantalizing connection to cell cycle-regulated proteolysis, lid1+p was found to encode a component of the anaphase-promoting complex (APC) or cyclosome (APC/C) (4).

The APC/C is a ubiquitin ligase required for regulated destruction of certain proteins during mitosis and G1 phase (reviewed in reference 41). It is a multisubunit complex that has been conserved throughout evolution. While the majority of subunits are stably associated throughout the cell cycle, the addition of transiently expressed CDC20 protein family members and posttranslational modifications activate the APC during mitosis and G1 phases (24). In S. cerevisiae and S. pombe, 13 core APC components have been identified through a combination of genetic and biochemical approaches (24, 40, 41).

We reported previously that Dim1p is required for maintaining the steady-state level of the APC component Lid1p and...
thus for maintaining the steady-state level and activity of the APC/C (4). We report here the results of our investigation into the mechanism whereby Dim1p influences Lid1p levels. We have found that S. pombe cells lacking Dim1p or S. cerevisiae cells lacking its ortholog, Dib1p, are defective in pre-mRNA splicing in vivo, a result consistent with the identification of Dim1p as a component of the purified yeast U4/U6.U5 tri-snRNP complex (14, 34). Moreover, we find that Dim1p can be copurified with the splicing factor Prp1p in a complex that is similar in composition to the human B31 complex. Since lid1 has four introns (4), the decrease in Lid1p levels we observed in the absence of Dim1p function might have been explained simply by defective pre-mRNA splicing. However, we provide evidence that this is not the full explanation for the dim1-35 phenotype and that Dim1p has roles in both lid1+ pre-mRNA splicing and the nuclear export of lid1+ mRNA.

**MATERIALS AND METHODS**

**Yeast methods, strains, and media.** S. pombe strains used in this study are listed in Table 1. Strains were grown in yeast extract medium or minimal medium with appropriate supplements (21). Crosses were performed on glutamate medium (minimal medium lacking ammonium chloride and containing 0.01 M glutamate [pH 5.6]). Random spore analysis and tetrad analysis were performed as described previously (21). Double mutant strains were constructed and identified by tetrad analysis. Unless otherwise indicated, transformations were performed by electroporation (29). The prp1+ gene was tagged at its chromosomal locus to encode a C-terminally tagged affinity purification (TAP)-tagged variant (35) by a PCR-mediated strategy as described previously (2). Proper integration of the TAP cassette was confirmed by PCR and immunoblotting.

**Construction of lid1+ expression vectors.** By using site-directed mutagenesis, Ndel and BamHI restriction sites were placed at the initiating methionine codon and just downstream of the stop codon of lid1+ in the genomic clone of lid1+ (pKg1295), and the four introns were removed from the lid1+ open reading frame (ORF) by site-directed mutagenesis to make pKg1430, a procedure performed by using a Bio-Rad Muta-Gen kit according to manufacturer’s instructions, to create the lid1Δ allele. The genomic and cDNA versions of the lid1+ ORF as Ndel-BamHI fragments were cloned downstream of the thiamine-repressible nmt1 promoter or its attenuated version, nmt1-41, in the vectors pREP1 (19) and pREP42HA, which add N-terminal hemagglutinin (HA) epitopes (12). All four vectors were able to rescue growth of lid1Δ and the lid1+ null mutant. Overexpression of lid1+ from these vectors was achieved by growth in the absence of thiamine, while repression was achieved by growth in the presence of 5 μg of thiamine/ml.

To introduce an N-terminal HA tag at lid1Δ at the lid1+ genomic locus, an Ndel fragment encoding three copies of the HA epitope was introduced at the Ndel site of pKg1430, and the lid1+ sequences were subcloned into the yeast expression vector pRT2 that carries the LEU2 selectable marker to make pKg2259. A diploid strain with the relevant genotype lid1Δ (lid1Δ::HA-lid1Δ) lid1-32 was transformed with this vector, and Ura+ colonies were selected and allowed to sporulate. Haploid progeny that were Ura− were isolated, grown to confluence in the absence of selection, and plated onto appropriate medium containing 5-fluoroorotic acid as described previously (10). Colonies that were Ura− were then isolated, and the correct replacement of the lid1:: arrogance locus with the epitope-tagged version of lid1+ was confirmed by PCR and Southern blotting.

**Plasmids and molecular biological techniques.** All plasmid manipulations and bacterial transformations were done according to standard techniques (32). Essential features of plasmid construction are described. All sequencing of plasmid DNA was performed by using Sequenase 2.0 (USB, Cleveland, Ohio) or Thermo Sequenase (Amersham Life Sciences, Cleveland, Ohio) according to the manufacturer’s instructions. PCR amplifications were performed by using Taq polymerase and Gene Amp reagents (Perkin-Elmer, Norwalk, Conn.). Pfu polymerase, BioExact (ISC BIOEXPRESS, Kaysville, Utah), or TaqPlus Precision (Stratagene, La Jolla, Calif.) according to the manufacturer’s instructions. Amplifications were accomplished by using a PTC-100 programmable thermal controller or a PTC-150 minicycler (MJ Research, Watertown, Mass.).

**Immunoprecipitations, immunoblots, and sucrose gradient sedimentation.** Protein lysates were made by glass bead disruption of the cell walls in a minimal volume of NP-40 buffer. For denatured lysates, lysed cells were heated to 95°C in sodium dodecyl sulfate (SDS) lysis buffer (10 mM NaPO4 [pH 7.4], 0.1% SDS, 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaF, 100 μM leupeptin/ml) for 2 min and extracted with NP-40 buffer (6 mM Na2HPO4, 4 mM NaH2PO4, 1.0% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 100 μM Na2VO4, 4 μg of leupeptin/ml) and protease inhibitors. For native lysates, heating in SDS lysis buffer was omitted. For immunoblots, a 1/5 volume of 5× sample buffer was added to the extracts. For quantitative immunoblots, denatured and clarified lysates were normalized by bicinchoninic acid assay (Pierce, Rockford, Ill.) so that equal amounts of protein were loaded into each well of 4 to 20% Tris-glycine polyacrylamide gel. Fractionated proteins were transferred onto Immobilon P membranes (Millipore Corp., Bedford, Mass.) and blotted with anti-Cdc5p (1/5,000), anti-Cdc3p (1/500), and anti-Cdc1p (1/500) rabbit polyclonal antisera and anti-Cdc2p PSTAIR monoclonal antibody (1/5,000) (Sigma, St. Louis, Mo.). 9E10 and 12CAS mouse monoclonal antibodies (1 μg/ml) were used to detect Myc- and HA-tagged proteins. Goat anti-rabbit and anti-mouse

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**Table 1. S. pombe strains used in this study**

| Strain designation | Genotype | Source or reference |
|--------------------|----------|---------------------|
| KGY28              | h+ 972   | P. Nurse            |
| KGY69              | h+ 975   | P. Nurse            |
| KGY246             | h+ leu1-3 ura4-D18 ade6-M210 | Lab stock |
| KGY390             | h+ dim1-35 ura4-D18 leu1-32 | Lab stock |
| KGY396             | h+ dim1-35 leu1-32 | Lab stock |
| KGY1088            | h+ pppl-myc::Kan1 ura4-D18 leu1-32 ade6-M210 | This study |
| KGY1147            | h+ pppl::TAP::Kan1 ura4-D18 leu1-32 ade6-M210 | This study |
| KGY1141            | h+ pppl-2 | This study |
| KGY1180            | h+ dim1::his3+ leu1-32:znmt1dim1+ his3-D1 ura4-D18 ade6-M210 | Lab stock |
| KGY1302            | h+ lid1-myc::Kan1 | Lab stock |
| KGY1305            | h+ lid1-myc::Kan1 dim1-35 | Lab stock |
| KGY1336            | h+ lid1-myc::Kan1 ura4-D18 leu1-32 ade6-M210 | Lab stock |
| KGY1365            | h+ cut9-HA::Kan1 | Lab stock |
| KGY1420            | h+ cfw3-myc::Kan1 leu1-32 ura4-D18 ade6-M210 his3-D1 | Lab stock |
| KGY1430            | h+ cfw2-myc::Kan1 leu1-32 ura4-D18 ade6-M210 his3-D1 | Lab stock |
| KGY1739            | h+ dim1-35 cut9-HA::Kan1 | This study |
| KGY1612            | h+ lid1::HA-lid1Δ dim1-35 leu1-32 ura4-D18 ade6-M21X | This study |
| KGY1858            | h+ dim1::HA::Kan1 leu1-32 ura4-D18 ade6-M210 | This study |
| KGY1455            | h+ ruel1-1 leu1-32 ura4-D18 | This study |
| KGY3210            | h+ dim1-35 cfw2-myc::Kan1 leu1-32 ura4-D18 ade6-M210 his3-D1 | This study |
| KGY3211            | h+ dim1-35 cfw2-myc::Kan1 leu1-32 ura4-D18 ade6-M210 his3-D1 | This study |
| KGY3514            | h+ lid1::HA-Hid1Δ leu1-32 ura4-D18 ade6-M21X | This study |
secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) were used at a 1/25,000 dilution. Proteins were visualized with the ECL+ detection system (Amersham) by fluorescence scanning (Storm PhosphorImager; Molecular Dynamics Inc., Sunnyvale, Calif.). 35S-labeled lysates were prepared in an identical manner except that cells were grown overnight in minimal medium and then grown for 4 h in the presence of 1 M of 35S-Trans label (ICN Pharmaceuticals, Costa Mesa, Calif.) prior to lysis.

Immunoprecipitations were performed for 1 h on ice followed by a 30-min incubation with 50 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia, Piscataway, N.J.). Immunoprecipitates were washed six times with NP-40 buffer and then resuspended in sample buffer. Anti-Myc immunoprecipitations were performed by using 5 μg of 9E10 antibody and 5 μg of rabbit anti-mouse antibody (Cappel; Orfanon Teknika Corp., West Chester, Pa.). Unless otherwise noted, 12CAS immunoprecipitations were performed by using 20 μg of 12CAS which had been coupled to protein A-Sepharose using dimethyl pimelimidate (Sigma). After 1.5 h of incubation, the immunoprecipitates were washed with NP-40 buffer and resuspended in sample buffer.

Proteins were resolved on SDS–6 to 20% polyacrylamide gels. For immunoblotting, proteins were then transferred by electroblotting onto a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp.). Epitope-tagged proteins were detected with 12CAS (to detect the HA tag) or 9E10 (to detect the Myc tag) antibody at 2 μg/ml in Tris-buffered saline followed by alkaline phosphatase-conjugated goat anti-mouse polyclonal antibodies (Sigma). Cdc2p was detected with anti-PSTAIRE antibodies (immunoblots were visualized by using enhanced chemiluminescence [ECL; Amersham]. For visualization of 35S-labeled proteins, the protein gels were fixed, treated for fluorography (Amplify; Amplify; Amersham), dried, and exposed to film.

Protein complexes were obtained by using the TAP strategy as described previously (35), except that the lysates were clarified at 3.000 rpm on a table-top GS-6r centrifuge in lieu of ultracentrifugation. TAP pellets were subjected to mass spectrometric analysis as described previously (8). Sucrose gradient sedimentation analysis was performed exactly as described previously (20), except that gradients were centrifuged for 16 h at 25,000 rpm.

RNA and Northern blots. Total RNA from S. pombe cells was prepared as described previously (12). The parS24 pressure dba1A suffixa strain (KGL1806) was grown under permissive conditions and shifted to restrictive conditions as described previously (5). The control mutants were ppp3-1 (ts125) (KLG1825) (7, 39), prp18 (ts101) (KGL1229) (39), and cdc28-1N (KGL760) (25). Total RNA was prepared from these cells by extraction with hot acidic phenol as described previously (11). To detect mRNAs, total RNA (20 μg) was resolved with formaldehyde agarose gels and capillary blotted to GeneScreen+ (Dupont-NEN; Boston, Mass.) or Duralon-UV (Stratagene). 523 DNA was detected by using 32P-labeled oligonucleotides complementary to both intronic (TFIHD I) and exonic (TFIHD E) sequences as described previously (17, 26). his3+ RNAs were detected by using the 32P-labeled EcoRv-dral segment of the genomic clone (10) as a probe. Blots of S. cerevisiae RNAs were hybridized with labeled probes from RPS51a, DNY2, and GLC7 ORFs or a PCR fragment representing the ACT1 intron sequences. Blots were exposed to Phosphorimager screens and visualized by using MD Image Quant software version 3.3 (Molecular Dynamics).

Microscopy. All microscopy was performed with a Zeiss Axioskop II equipped with a z-focus motor drive, and images were captured with an Orca II charge-coupled-device camera (Hamamatsu, Japan). Images were obtained, processed, and analyzed with OpenLab 2.1.3 software (Improvision, Lexington, Mass.).

In situ hybridization. Cells were fixed in suspension with 3.7% formaldehyde (for 30 min, washed twice in 0.1 M potassium phosphate, pH 6.5 (K-Pi buffer)), washed once in K-Pi buffer plus 1.2 M sorbitol (K-Pi/SORB), and resuspended in 1 ml of a 1:1 slurry of protein A-Sepharose (Pharmacia, Piscataway, N.J.). Cells were then resuspended in a solution containing 100 μl of 50% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt’s solution, 125 μg of tRNA/ml, 10% dextran sulfate, and 500 μg of denatured salmon sperm DNA/ml and incubated for 1 h at 37°C. A 418-ppm oligo(dT)10 probe, 3’-end labeled with digoxigenin-11-dUTP (Boehringer Mannheim) as previously described (13), was added to the cells, and they were incubated overnight at 37°C while rotating. The cells were then washed for 1 h in 2× SSC at room temperature, 1 h in 1× SSC at room temperature, 30 min in 0.5× SSC at 37°C, 30 min in 0.5× SSC at room temperature, 5 min in phosphate-buffered saline containing 1% bovine serum albumin (PBAL) at room temperature, and 1 h in PBAL at room temperature. Cells were resuspended in 50 μl of PBAL, and rabbit polyclonal anti-digoxigenin antibody conjugated to fluorescein isothiocyanate was added at a dilution of 1:25 and then incubated for 1 h at room temperature. Cells were subsequently washed two to three times in PBAL and mounted onto slides as described previously (13).

Yeast two-hybrid analysis. The yeast two-hybrid system used in this study was described previously (15). The indicated cDNAs were cloned into the bait plasmid pGB7 and/or the prey plasmid pAD424 (Clontech, Palo Alto, Calif.) and sequenced to ensure the absence of PCR-induced mutations and to ensure that the correct reading frame had been retained. To test for protein interactions, both bait and prey plasmids were cotransformed into S. cerevisiae strain PJ69-4A. β-Galactosidase reporter enzyme activity in the two-hybrid strains was measured by using the Galacto-Strat chemiluminescent reporter assay system according to the manufacturer’s instructions (Tropix Inc., Bedford, Mass.), with the exception that cells were lysed by glass bead disruption. Each sample was measured in triplicate. Reporter assays were recorded on the Mediators Phl. luminometer (Aurion Biosystems, Vienna, Austria).

Green RNA. The system for imaging the localization of specific mRNA transcripts was adapted from a similar system in S. cerevisiae (a generous gift of Kelly Bloom [3]). The green fluorescent protein (GFP)-fused MS2 coat protein (CP-CP-GFP) was subcloned by PCR from pCP-GFP into the NdeI/NotI sites of pREP4INT (35), creating the pREP41CP-GFP vector. Primers used to amplify CP-GFP were pCP-GFP-For (5’T-TAGGGCCCGCCTATATGTCCTAATCT TACTCAGTGTTCCTCCTCGC-3’) and pCP-GFP-Rev (5’T-TTCTTTTGTG GCGCCCGGGTGACTATTGTTATGATTGTG-3’). To create a host vector for RNA transcripts of interest, an attenuated adh1 promoter (a generous gift of Charlie Albritton) was subcloned from pSK utilizing PstI/Xhol and ligated into the pREP2 vector (19) previously digested with PstI/Sall, creating the pRAM vector (pREP with adh1 mutated). The 154-bp MS2 binding site sequence was excised from pIIIa/MS2-2 by digestion with EcoRI. This fragment contains two tandem copies of the 25-nucleotide MS2 coat protein binding site and a single adjacent Smal site for cloning sequences of interest. The fragment was first treated with Klenow fragment to create blunt ends and subsequently ligated into the Smal site of pRAM to create pRAM-MS2. All sequences examined were cloned into the Smal site of pRAM-MS2, and visualization was accomplished by cotransformation with pREP41CP-GFP and examination of GFP fluorescence in live cells of the strains indicated.

RESULTS

dim1+ function is required for pre-mRNA splicing. To learn more about the biochemical function of Dim1p, we examined the biochemical basis for the decreased abundance of the APC/C component Lid1p in the absence of dim1+ function. Because Lid1p is involved in the process of proteolysis, we began our analysis by examining Lid1p degradation rates in the presence and absence of dim1+ function. For this purpose, the lid1+ ORF including its four introns was tagged with sequences encoding three copies of the HA epitope at its 5’ end, and the fusion construct was placed under control of the thiamine-repressible attenuated nmt1 promoter (nmt41). The plasmid expressing HAld1+ was able to rescue both the lid1-6 and lid1 null alleles, and overexpression elicited no obvious phenotype in any background (data not shown). HAld1+ was then overexpressed in wild-type and dim1-35 cells. Although HA-Lid1p was readily detected in lysates prepared from wild-type cells, it was present at significantly reduced levels in dim1-35 cells even when the cells were grown at 25°C, a temperature fully permissive for growth (Fig. 1A). This result reproduces what was observed previously for endogenous Lid1p in the absence of dim1+ function (4).

Despite the lower level of total HA-Lid1p in dim1-35 cells compared to wild-type cells, we were able to determine the relative half-life of HA-Lid1p in the two strains. After maximal expression was achieved by growing cells in the absence of thiamine, synthesis of the RNA and protein was repressed by adding thiamine and cycloheximide, respectively, to the me-
dium. The amount of HA-Lid1p that remained was determined at hourly time points by immunoblotting with antibodies to the HA epitope. In wild-type cells, after an initial burst of protein production, the amount of HA-Lid1p declined steadily over the course of the experiment (Fig. 1B, upper panel). Although expressed at significantly reduced levels from the nmt41 promoter, equal numbers of cells were collected at hourly intervals, and protein lysates were prepared. Proteins were resolved by SDS-PAGE. HA-Lid1p was detected with 12CA5 antibodies, and Cdc2p, which served as a loading control, was detected with anti-PSTAIR monoclonal antibody. The immunoblot in the lower panel was developed for longer than the immunoblot in the upper panel so that the lower level of HA-Lid1p in dim1 cells could be visualized throughout the course of the experiment.

Based on this result, we then predicted that HA-Lid1p was synthesized at reduced levels in the absence of dim1 function. To test this hypothesis, wild-type and dim1 cells expressing maximal levels of HA-Lid1p were pulsed for 10 min with 35S-S-Trans label, and the amount of HA-Lid1p produced was determined by immunoprecipitation. Despite similar amounts of 35S incorporation into the strains during a 10-min pulse, there was significantly less HA-Lid1p produced in dim1 cells than that produced in wild-type cells (data not shown).

We next examined the steady-state level of HA-lid1 mRNA produced from the nmt41 promoter in dim1 cells relative to wild-type cells by Northern blot analysis. Although the total levels of nmt41 HA-lid1 mRNA production were similar at both permissive and nonpermissive temperatures, the nmt41 HA-lid1 RNA was split into two bands in the dim1 cells (Fig. 2A, strain 3). The faster-migrating band comigrated with the nmt41 HA-lid1 RNA from wild-type cells. Since lid1 contains four introns, it seemed likely that the upper band represented an unspliced form of nmt41 HA-lid1 RNA. To test this possibility directly, the four introns were removed from the nmt41 HA-lid1 construct. When the cDNA version of lid1 was presented an unspliced form of RNAs were detected with a 32P-labeled probe derived from the mature ORF as probe. Note by the absence of bands in 1 and 2 that endogenous levels of lid1 RNA are not detected in these exposures. B) Total RNA was purified from wild-type (wt), prp2-1 shifted to the nonpermissive temperature for the indicated number of hours, a strain containing dim1::his3 and nmt1-T81 dim1 integrated at the leu1 locus grown in presence of thiamine for the indicated number of hours, and dim1 cells shifted to the nonpermissive temperature for the indicated number of hours. Twenty micrograms of total RNA from each sample was resolved by electrophoresis and subjected to Northern blot analysis with oligonucleotide probes complementary to the intron and exon sequences within the lid1 ORF. Note the mature form of GLC7 mRNA does not decline because of the presumed longer half-life of the species.

FIG. 1. HA-Lid1p cannot be overproduced in the absence of dim1 function. Wild-type and dim1 cells were transformed with pREP42HA-lid1. Transformants were grown at 25°C in the absence of thiamine for 20 h, and samples were collected. A) Total protein lysates were prepared from the samples and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Following immunoblotting with 12CA5 to detect HA-Lid1p and anti-Arp3p serum (19a) to detect Arp3p as a loading control, proteins were visualized by enhanced chemiluminescence. B) After maximal induction of pREP42HA-lid1 in wild-type (upper panel) and dim1 (lower panel) cells, 5 μg of thiamine/ml was added to the medium to prevent further expression from the nmt41 promoter. Equal numbers of cells were collected at hourly intervals, and protein lysates were prepared. Proteins were resolved by SDS-PAGE. HA-Lid1p was detected with 12CA5 antibodies, and Cdc2p, which served as a loading control, was detected with anti-PSTAIR monoclonal antibody. The immunoblot in the lower panel was developed for longer than the immunoblot in the upper panel so that the lower level of HA-Lid1p in dim1 cells could be visualized throughout the course of the experiment.

FIG. 2. Cells lacking dim1 function are defective for pre-mRNA splicing. A) The lid1 ORF with (strains 3 and 4) or without (strains 5 and 6) its four introns was introduced into the pREP42HA vector. Wild-type cells (strains 2, 4, and 6) and dim1 cells (strains 1, 3, and 5) were transformed with vector alone (strains 1 and 2) or the pREP42HA-lid1 constructs (strains 3 to 6). Transformants were grown at 25°C in the absence of thiamine for 18 h and then transferred to 36°C for 0 or 4 h. Total RNA was prepared and subjected to Northern analysis with a fragment of the lid1 ORF as probe. Note by the absence of bands in 1 and 2 that endogenous levels of lid1 RNA are not detected in these exposures. B) Total RNA was purified from wild-type (wt), prp2-1 shifted to the nonpermissive temperature for the indicated number of hours, a strain containing dim1::his3 and nmt1-T81 dim1 integrated at the leu1 locus grown in presence of thiamine for the indicated number of hours, and dim1 cells shifted to the nonpermissive temperature for the indicated number of hours. Twenty micrograms of total RNA from each sample was resolved by electrophoresis and subjected to Northern blot analysis with oligonucleotide probes complementary to the intron and exon sequences within the lid1 ORF. Note the mature form of GLC7 mRNA does not decline because of the presumed longer half-life of the species.

S. cerevisiae cells lacking DIB1 are defective in pre-mRNA splicing. Strain KGY1023 was maintained in synthetic medium containing rafose and galactose. DIB1 expression was repressed by shifting the cells to synthetic medium containing glucose (SD). Aliquots of cells were collected at the number of hours indicated following the shift into synthetic medium containing glucose. Total RNA was also purified from temperature-sensitive mutants prp3-1, prp18 (ts503), and cdc28-1N shifted to the restrictive temperature (35.5°C) for the number of hours indicated. Twenty micrograms of total RNA was electrophoresed and blotted. Northern blots probed with the ACT1 intron sequence or the DYN2, GLC7, and RP51a ORFs. Note the mature form of GLC7 mRNA does not decline because of the presumed longer half-life of the species.
overexpressed in dim1-35 cells in parallel with the intron-containing form, the upper band was no longer observed (Fig. 2A, strain 5). Thus, dim1-35 cells appeared to be defective in the splicing of lid1+ pre-mRNA.

The failure of dim1-35 cells to splice lid1+ mRNA might explain the reduced levels of Lid1p in this strain. To test whether Dim1p was required for general pre-mRNA splicing in vivo, RNA was prepared from wild-type cells, prp2-1 (a bona fide pre-mRNA splicing mutant), cells, cells genetically depleted for dim1+ (a dim1 null mutant containing nmt1-regulatable dim1+), and dim1-35 cells, and the RNA was subjected to northern analysis using probes directed at two intron-containing genes, tf2d and his3+. Like prp2-1 cells, cells lacking dim1 function accumulated unspliced RNAs (Fig. 2B).

We next tested whether the S. cerevisiae ortholog of Dim1p, termed Dib1p, was required for pre-mRNA splicing, since Dib1p had also been implicated in pre-mRNA splicing due to its copurification with the U4/U6.U5 tri-snRNP (14, 34). Previously, in order to investigate the phenotype of cells lacking Dib1p function, we had created a conditional expression strain, KGY1023 (5). Because GAL1-driven DIB1 was not sufficiently repressed by glucose addition, we made use of the ubiquitin-N-degron tagging strategy described previously by Althoefer et al. (1). A plasmid expressing budding yeast DIB1 still allowed growth in the presence of glucose. However, a single integrated copy of GALS::UBdim1+ rescued growth under inducing conditions but failed to rescue growth under repressing conditions. Both S. pombe dim1+ and mouse mDim1 rescue the dib1 null mutation (5). KGY1023, which lacks endogenous DIB1 and harbors plasmid-borne S. pombe dim1 cDNA under control of the GAL1 promoter, arrests growth following 6 h of glucose repression (5). KGY1023 mRNA was compared to that isolated from three control strains: (i) prp3-1, a positive control for a defect in the first step of splicing (39); (ii) prp18 (ts503), a positive control for a defect in the second step of splicing (39); and (iii) cdc28-1N, a G1 arrest (25) control to ensure that any observed splicing defects were not secondary to cell cycle arrest. We assayed four intron-containing transcripts: ACT1 and RP51a, which are routinely used to analyze splicing defects in prp mutants; DYN2, a transcript in S. cerevisiae that contains two introns; and GLC7, which encodes a cell cycle-regulated protein. When dim1 expression was repressed, intron-containing forms of all four transcripts steadily accumulated to levels comparable to what was observed in the prp mutants (Fig. 2C). Also, the levels of mature DYN2 and RP51a decreased throughout the time course. These results were comparable to those observed with the prp3-1 mutant but distinct from those observed with prp18 and cdc28-1N. These data therefore suggest that DIB1 is essential, either directly or indirectly, for the first step of pre-mRNA splicing in vivo.

**Dim1p copurifies with known splicing factors.** To determine whether Dim1p was a part of the S. pombe U4/U6.U5 tri-snRNP, similar to Dib1p in S. cerevisiae (14, 34), we examined whether it was present in a high-molecular-weight complex by sucrose gradient sedimentation. We found that a percentage of Dim1p sedimented deeper into the gradient than Cdc5p (which runs at approximately 40S) (20) (Fig. 3A), indicating that Dim1p was present in a complex considerably larger than what would be expected from the tri-snRNP (34). The remainder of Dim1p sedimented near the top of the gradient, consis-
as were most components of the U4/U6.U5 tri-snRNP and the U2 snRNP. Indeed, the protein composition of this complex is very similar to the recently described human BA1 complex that lacks the U1 snRNP and the nineteen complex (18). The amino acid sequence coverage of Prp1p was 60%, and the greatest sequence coverage of copurifying proteins was obtained for Dim1p and Prp31p, at 58 and 61%, respectively. While these results are not quantitative, they did raise the possibility that Dim1p interacted with Prp1p directly or indirectly through an association with Prp31p, and we tested whether Dim1p was capable of binding either of these two proteins. We found that Dim1p interacted with Prp1p by two-hybrid analysis using full-length constructs of each protein, but it did not show an interaction with Prp31p (Fig. 3C). In contrast, Prp1p showed a strong interaction with both Dim1p and Prp31p (Fig. 3C and D), indicating that Prp1p might bind both proteins directly and simultaneously.

The dim1Δ35 mutation selectively affects the production of Lid1p. A block to pre-mRNA splicing as in the dim1Δ35 mutant would be expected to affect the levels of most transcripts and their protein products. However, this seemed incongruous for two reasons. First, we had used Cdc2p and Arp3p as loading controls for our immunoblots and observed no difference in their abundance between wild-type and controls for our immunoblots and observed no difference in

### TABLE 2. Comparison of mass spectrometric results from protein purifications of S. cerevisiae penta-snRNP, S. pombe Cdc5-TAP, and S. pombe Prp1-TAP

| S. pombe | S. cerevisiae | Human | 1st | 2nd | 3rd |
|----------|---------------|-------|-----|-----|-----|
| Smb1p    | Smblp         | SmBlp | X   | X   | X   |
| Smldp    | Smldlp        | SmDlp | X   | X   | X   |
| Cfwp9p   | Sm2dp         | Sm2dp | X   | X   | X   |
| Smldp    | Sm3dp         | Sm3dp | X   | X   | X   |
| Smldp    | Smldlp        | SmE1p | X   | X   | X   |
| Smldp    | Smldlp        | SmF1p | X   | X   | X   |
| Smldp    | SmG1lp        | SmG1p | X   | X   | X   |
| Lsm2p    | Lsm2p         | LSM2  | X   | -   | -   |
| Lsm3p    | Lsm3p         | LSM3  | X   | -   | -   |
| Lsm4p    | Lsm4p         | LSM4  | X   | -   | -   |
| Lsm5p    | Lsm5p         | LSM5  | X   | -   | -   |
| Lsm6p    | Lsm6p         | LSM6  | X   | -   | -   |
| Lsm7p    | Lsm7p         | LSM7  | X   | -   | -   |
| Lsm8p    | Lsm8p         | LSM8  | X   | -   | -   |
| U1 snRNP proteins
| U1-70    | Snplp         | U1-70 | X   | -   | -   |
| U1-A     | Mudlp         | U1-A  | X   | -   | -   |
| U1-C     | Yhclp         | U1-C  | X   | -   | -   |
| Prp40p   | Prp40p        |       | X   | -   | -   |
| Prp39p   | Prp39p        |       | X   | -   | -   |
| SPBC23E6.01c | Namlp  | X   | -   | -   |
| Llc7p    | Llc7p         | X     | -   | -   |
| Smb1p    | Smblp         |       | X   | -   | -   |
| SPBC24E9.10 | Sm2lp  | X   | -   | -   |
| U2 snRNP proteins
| U2Ap     | Lealp         | U2A   | X   | X   | X   |
| O13649   | Msilp         | U2-A  | X   | X   | X   |
| Sap1p    | Prp9p         | SFa60 | X   | X   | X   |
| Sap2p    | Prp11p        | SFa66 | X   | X   | X   |
| Sap114p  | Prp12p        | SFa120| X   | X   | X   |
| Sap49p   | Hsb49p        | SFb53 | X   | X   | X   |
| Sap145p  | Cus1p         | SFb150| X   | X   | X   |
| Prp12p   | Rce1p         | SFb130| X   | X   | X   |
| Prp10p   | Hsh155p       | SFb160| X   | X   | X   |
| Uap2p    | Cus2p         | Tat-561| X | X   |
| SPBC29A3.07c |       | p14  | -   | -   | X   |
| O94290   | 1st3p/Snu17p  |       | X   | X   |
| U5 snRNP proteins
| Cw66p    | Prp8p         | U5-220| X   | X   | X   |
| Br12p    | Br12p         | U5-200| X   | X   | X   |
| Cw10lp   | Smu114p       | U5-116| X   | X   | X   |
| Prp28p   | Prp28p        | U5-100| X   | X   | X   |
| Cw17p    | Prp40p        | U5-40  | -   | X   | X   |

* Tri-snRNP proteins

| S. pombe | S. cerevisiae | Human | 1st | 2nd | 3rd |
|----------|---------------|-------|-----|-----|-----|
| Prp1p    | Prp6p         | U5-102| X   | -   | X   |
| Q09856   | Prp3p         | HPR3  | X   | -   | X   |
| Q9UTC7   | Prp4p         | HPR4  | X   | -   | X   |
| Sm66p    | Smu66p        | SART1 | X   | -   | X   |
| Prp31p   | Prp31p        | PRP31 | X   | -   | X   |
| SFAC507.03c | Snu13p   | SNU13 | X   | -   | X   |
| Snu23p   | Prp38p        |       | X   | -   | X   |
| Prp38p   | Snp38p        |       | X   | -   | X   |
| Q9USR2   | Sad1p         | Q96RK9| X   | -   | X   |
| Dimlp    | Diblp         | U5-15  | X   | X   |

* Ntc proteins

| S. pombe | S. cerevisiae | Human | 1st | 2nd | 3rd |
|----------|---------------|-------|-----|-----|-----|
| Cdc5p    | Ceflp         | CDC5L | X   | -   | X   |
| Prp5p/Cw1lp | Prp46p     | PRL1  | X   | -   | X   |
| Cw2lp/Pp3lp | Cw2cp       | RNPS1 | X   | -   | X   |
| Cw3p     | Sy1p          | SYF1  | X   | -   | X   |
| Cw4p     | Sy1p          | CRN1  | X   | -   | X   |
| Cw7p     | Sm309p        | SPF27 | X   | -   | X   |
| Cw8lp    | Prp10p        | PRP19 | X   | -   | X   |
| Cw11lp   | Is1p          | ISY1  | X   | -   | X   |
| Cw13lp   | Prp45p        | SK1P  | X   | -   | X   |
| O59733   | Sy1lp         | GCIP-IP| X   | -   | X   |

Second-step factors

| S. pombe | S. cerevisiae | Human | 1st | 2nd | 3rd |
|----------|---------------|-------|-----|-----|-----|
| Q9Y7Y2   | Shulp         | SLU7  | X   | -   | X   |
| Cw5p     | St1lp         | ECM2  | X   | -   | X   |
| Prp17p   | Prp17p        | PRP7  | X   | -   | X   |
| Prp22p   | Prp22p        | PRP22 | X   | -   | X   |

Other factors

| S. pombe | S. cerevisiae | Human | 1st | 2nd | 3rd |
|----------|---------------|-------|-----|-----|-----|
| Cw11p    |               | O60306| X   | -   |
| Cw15p    | Cwc15p        | HSPC148| X   | -   |
| Cw21p    |               | SRM300| X   | -   |
| Cw22p    | Cwc22p        | M1F4G | X   | -   |
| Cw24p    | Cwc24p        | O15541| X   | -   |
| Cw27p    | Cwc27p        | PPPL1 | X   | -   |
| O43031   | Spp2p         | Q9BQA8| X   | -   |
| Cw14p    | Cwc14p        | G10   | X   | -   |
| Cw16p    | Cwc16p        | Q9BWB8| X   | -   |
| Cw18p    |               | MGCG23918| X   | -   |
| Cw19p    |               |       | X   | -   |
| Cw22p    |               |       | X   | -   |

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1A), although both the cdc2+ and arp3+ primary transcripts contain four introns. Second, it would be difficult to imagine a scenario in which a general block in protein production would generate the dim1-35 mutant phenotype. To examine this question more carefully, we compared the levels of several proteins whose pre-mRNAs contain introns in the dim1-35 mutant and wild-type cells. The levels of Cdc3p (profilin), Cdc4p (a myosin light chain), Cdc5p (a pre-mRNA splicing factor), Arp3p (a component of the Arp2/3 F-actin-nucleating complex), Ctf6p (a pre-mRNA splicing factor), and Cwf2p (a pre-mRNA splicing factor) were not significantly different in the dim1-35 mutant relative to wild-type cells using the amount of Cdc2p in the lysates as a loading control (Fig. 4A). Only Lid1p levels were found to be significantly different (Fig. 4A). This was also true if the specific signals were quantitated against total protein loaded onto the gels rather than Cdc2p abundance (data not shown). Thus, the dim1-35 mutation selectively affects the production of Lid1p among tested proteins.

If Dim1p controlled the levels of Lid1p solely by regulating the splicing of lid1+ RNA, then production of Lid1p in dim1-35 cells should be restored to wild-type levels by the removal of the four introns from the lid1+ ORF. To test this hypothesis, we examined the level of HA-Lid1p produced from the lid1+ cDNA (lid1Δi) under control of the nmt41 promoter. Unexpectedly, we found that HA-Lid1p levels were still reduced in dim1-35 cells relative to that of wild-type cells, although the level of Cdc2p did not vary (Fig. 4B, lanes 5 and 6). We then considered the possibility that overproduction of HA-Lid1p from a heterologous promoter was overwhelming the capacity of dim1-35 cells to produce HA-Lid1p. Therefore, we constructed a gene replacement strain. First, we introduced sequences encoding three copies of the HA epitope at the 5’ end of the open reading frame of the intron-deleted version of lid1+. Next, the tagged version of lid1Δi was used to replace the endogenous gene (see Materials and Methods) so that expression would occur from the endogenous lid1 promoter. The HA-Lid1Δi strain was wild type in morphology and growth rate (data not shown). The HA-Lid1Δi allele was then combined with the dim1-35 mutation, and endogenous HA-Lid1p levels were examined after a shift to a restrictive temperature. The amount of HA-Lid1p produced in dim1-35 cells was barely detectable and significantly less than that in wild-type cells (Fig. 4C). Thus, there appears to be a second block to Lid1p production downstream of pre-mRNA splicing in dim1-35 cells.

Dim1p function is required for efficient pre-mRNA export.

The process we thought to examine next in cells lacking dim1+ function was the export of RNA from the nucleus. The localization of poly(A)+ RNA was examined in dim1-35 and dim1Δ cells and compared with that in wild-type cells and a bona fide nuclear export mutant, rae1-1 (9). In wild-type cells, poly(A)+ RNA was not detected in the nucleus at appreciable levels (Fig. 5A). In the dim1-35 mutant, poly(A)+ RNA could be detected in the nucleus even at permissive temperature, and staining within the nucleus increased during the temperature shift (Fig. 5A). However, nuclear accumulation was neither as complete nor as rapid as that observed in rae1-1 cells (Fig. 5A). In the dim1Δ cells maintained by dim1+ expressed from a regulatable thiamine-repressible promoter, nuclear pre-mRNA accumulation was observed concomitantly with the timing of promoter repression (Fig. 5B). Furthermore, this accumulation paralleled the timing of the loss of pre-mRNA splicing (Fig. 5C).

The dim1-35 mutation selectively affects the export of lid1+ mRNA.

One possible explanation for the selective effects of Dim1p loss of function on Lid1p levels is that the function of Dim1p in either pre-mRNA splicing and/or nuclear mRNA export is specific to lid1 transcripts or a subset of transcripts that includes lid1. Since our data indicated a generalized defect of pre-mRNA splicing (Fig. 2), we examined the specificity of the dim1-35 mRNA export defect for lid1 transcripts. To address this, we adapted the S. cerevisiae “green RNA” system, developed for live cell monitoring of specific mRNA transcripts (3, 33), for use in S. pombe (see Materials and Methods). In wild-type cells, the lid1+ GFP-labeled mRNA transcript (gRNA) was not detected in the nucleus at appreciable levels (Fig. 6). However, in the dim1-35 mutant, lid1+ gRNA could be detected in the nucleus at the restrictive temperature (Fig. 6). However, as observed with total mRNA (Fig. 5), nuclear accumulation was not as complete or as rapid as that.
observed in rae1-1 cells (Fig. 6). The accumulation of lid1 transcript in dim1-35 is unrelated to the role of Dim1p in splicing, as both wild-type and lid1Δ transcripts showed comparable levels of accumulation. Furthermore, no appreciable accumulation of lid1 transcript was observed in prp2-1 cells, which are strongly inhibited for pre-mRNA splicing, at the restrictive temperature (data not shown). To investigate the specificity of the RNA export defect, we also examined the localization of the unrelated wsp1 transcript. While wsp1 transcripts with or without introns do accumulate in the nucleus of rae1-1 cells (Fig. 6 and data not shown), which show a block of generalized mRNA export (9), no significant accumulation of these transcripts was observed in dim1-35 cells (Fig. 6).

**DISCUSSION**

In this study, we have investigated the observed dependence of Lid1p protein levels on Dim1p function. Consistent with previous observations that Dim1p orthologs associate with splicing factors (14, 34, 43), we have found that *S. pombe* Dim1p is required for efficient pre-mRNA splicing. However, our data indicate that Dim1p’s essential function extends be-
yond pre-mRNA splicing to mediating the export of at least certain mRNAs from the nucleus.

While Dim1p orthologs have previously been purified along with the U4/U5.U6 tri-snRNP (14, 34), we purified *S. pombe* Dim1p with the U4/U5.U6 tri-snRNP component Prp1p (equivalent of Prp6 in *S. cerevisiae*) in a large splicing complex that appears similar in protein composition to the recently described B/H11001 complex isolated from human splicing extracts (18). Indeed, by sucrose gradient fractionation, we did not detect a smaller Prp1p-containing protein complex. The Prp1p-TAP complex contained U4/U5.U6 tri-snRNP and U2 snRNP components, while it lacked any detectable contribution of the nineteen complex that is a hallmark of the U2,U5,U6 complex that has predominated purification of splicing complexes from *S. pombe* (23). While Dim1p was identified within this large protein complex, fractionation of *S. pombe* lysates by sucrose gradient sedimentation indicates that Dim1p/Dib1p exists outside of this splicing complex as well, most likely in smaller complexes or on its own. Thus, our biochemical analyses leave open the possibility that Dim1p/Dib1p performs functions outside of the U4/U5.U6 tri-snRNP and possibly in processes other than pre-mRNA splicing.

Because Dib1p was initially copurified with a much smaller U4/U5.U6 tri-snRNP *S. cerevisiae* complex, it seems likely that it interacts directly with at least one U4/U5.U6 tri-snRNP component. Indeed, in a genome-wide two-hybrid analysis of *S. cerevisiae* protein interactions, Dib1p was found to interact only with Prp6p, and Prp6p interacted only with Dib1p (36). The human homolog of Prp6p has also been shown to interact with the human homolog of Prp31p (18a). We have established the conservation of these interactions by showing that *S. pombe* Dim1p interacts with Prp31p, Prp1p contains many TPR repeats, and it will be interesting to narrow down the domain responsible for Dim1p and Prp31p interactions in the future. The structure of Dim1p family proteins has been determined by both nuclear magnetic resonance (42) and X-ray crystallography (31), and it has previously been suggested that the key role of these proteins in splicing complexes might involve binding of RNA via a conserved basic patch on their surfaces (43). However, we have been unable to detect any direct interactions between Dim1p and numerous RNA species (data not shown). Therefore, the basic patch region may be critical for a protein-protein interaction with an acidic region of the Prp1p-Prp31p splicing complex.

Since the completion of the *S. pombe* genome sequence, it has become clear that ~45% of *S. pombe* genes contain introns, and therefore, it is unexpected that a mutation in a protein required for general pre-mRNA splicing would have a very specific defect in the metaphase-to-anaphase transition due to inadequate production of a single component of the APC, Lid1p/Apc4p. The *S. pombe* APC contains 13 components, and several of these components are produced from genes containing introns. Of these proteins, however, only Lid1p levels fall significantly in the absence of Dim1p function (4; our unpublished data). This might indicate that *dim1* is a hypomorphic mutant that allows significant pre-mRNA splicing to occur. In this scenario, only the levels of short-lived, low-abundance proteins or RNAs would be expected to change dramatically within a 4-h temperature shift experiment. The scarcity of Lid1p combined with its requirement for APC function might make it an ideal target for regulation of the cell cycle via an arrest in proper mRNA processing. Indeed, our data suggest that at least in the case of Dim1p, this regulation is directed rather specifically towards Lid1p. Alternatively, and because we found it difficult to envision that *lid1* would surface as the single most critical low-abundance message or target, we have entertained possible explanations for the *dim1-35*
mutant phenotype other than a block to pre-mRNA splicing. Clearly, our biochemical fractionation results showing that a substantial fraction of Dim1p is not a part of a splicing complex and that lid1 RNA localization results are compatible with a specific requirement for Dim1p in other steps of pre-mRNA processing. It is also intriguing to us that prp1 and dim1 mutants display similar phenotypes. Like dim1-35 cells, prp1 mutants have been shown to have defects in pre-mRNA splicing, poly(A)+ RNA nuclear transport, and cell cycle control (26, 27, 28, 37, 38). This finding suggests that a complex containing Dim1p and Prp1p, and perhaps other proteins, might be critical in the transition steps between pre-mRNA splicing and transport of the mature transcript from the nucleus to the cytoplasm. These effects on RNA export are unlikely to be secondary effects related to defects in splicing, as the two defects are detected roughly simultaneously in dim1 mutant cells.

While undertaking these studies, we have generated the first system for real-time imaging of specific RNAs in S. pombe. This S. pombe green RNA system should be of use in future studies to define additional factors involved in RNA processing and export.

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