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A Connection between Pre-mRNA Splicing and the Cell Cycle in Fission Yeast: *cdc28*$^+$ Is Allelic with *prp8*$^+$ and Encodes an RNA-dependent ATPase/Helicase

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The fission-yeast gene *cdc28*$^+$ was originally identified in a screen for temperature-sensitive mutants that exhibit a cell-division cycle arrest and was found to be required for mitosis. We undertook a study of this gene to understand more fully the general requirements for entry into mitosis. Cells carrying the conditional lethal *cdc28-P8* mutation divide once and arrest in G2 after being shifted to the restrictive temperature. We cloned the *cdc28*$^+$ gene by complementation of the temperature-sensitive growth arrest in *cdc28-P8*. DNA sequence analysis indicated that *cdc28*$^+$ encodes a member of the DEAH-box family of putative RNA-dependent ATPases or helicases. The Cdc28 protein is most similar to the Prp2, Prp16, and Prp22 proteins from budding yeast, which are required for the splicing of mRNA precursors. Consistent with this similarity, the *cdc28-P8* mutant accumulates unspliced precursors at the restrictive temperature. Independently, we isolated a temperature-sensitive pre-mRNA splicing mutant *prp8-1* that exhibits a cell-cycle phenotype identical to that of *cdc28-P8*. We have shown that *cdc28* and *prp8* are allelic. These results suggest a connection between pre-mRNA splicing and progression through the cell cycle.

INTRODUCTION

Intensive genetic and biochemical studies of the cell-division cycle have converged to give a relatively cohesive picture of the major eukaryotic cell-cycle regulators.[1] In yeasts, control of the cell cycle is regulated primarily by the activity of the protein kinase p34^{cdc2} (Draetta, 1990; Nurse, 1990). This activity is, in turn, regulated by physical association with a family of proteins known as cyclins (Draetta, 1990; Pines and Hunter, 1991) and by phosphorylation and dephosphorylation at specific and critical points in the cell cycle (Draetta *et al.*, 1988; Morla *et al.*, 1989; Ducommun *et al.*, 1991; Gould *et al.*, 1991).

Many cell-cycle regulators have been identified in genetic screens for cell-division cycle (*cdc*) mutants in the budding yeast *Saccharomyces cerevisiae* (Hartwell, 1978) and the fission yeast *Schizosaccharomyces pombe* (Nurse, 1975). These mutations define genes whose products are required for progression through the cell cycle. In many cases these proteins have been shown to have conserved functions in all eukaryotes. The *S. pombe cdc2*$^+$ gene was identified in such a screen and shown to be required at two points in the cell cycle: in G1, before start, and in G2 (Nurse and Bissett, 1981). Several genes have been identified that regulate p34^{cdc2} activity. These in-

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clude *wee1*$^+$ and *mik1*$^+$, a pair of tyrosine kinases, and *cdc25*$^+$ and *pyp3*$^+$, a pair of tyrosine phosphatases, which function antagonistically to regulate the phosphorylation state of p34^{cdc2} (Russell and Nurse, 1986, 1987; Lundgren *et al.*, 1991; Millar *et al.*, 1992). In addition, four cyclins —*cdc13*$^+$, *puc1*$^+$, *cig1*$^+$, and *cig2*$^+$ —that function as regulatory subunits of the p34^{cdc2} protein kinase have been identified (Booher and Beach, 1987, 1988; Bueno *et al.*, 1991; Bueno and Russell, 1993; Forsburg and Nurse, 1991; Connolly and Beach, 1994).

Other *cdc* genes in fission yeast define functions that, although not directly related to regulation of p34^{cdc2}, must be executed for the cell cycle to progress. Examples include *cdc17*$^+$, the gene for DNA ligase (Nasmyth, 1977), *cdc22*$^+$ and *suc22*$^+$, which encode the large and small subunits of ribonucleotide reductase (Fernandez Sarabia *et al.*, 1993), and *cdc10*$^+$ and *sct1*$^+$, which encode components of a transcription complex required for commitment to the cell-division cycle at start (Aves *et al.*, 1985; Lowndes *et al.*, 1992; Caligiuri and Beach, 1993). Thus, mutations in genes that regulate p34^{cdc2} as well as in genes that participate in general cellular functions, such as DNA synthesis and transcription, can elicit *cdc* phenotypes.

Mutations in genes involved in RNA metabolism can also affect cell-cycle progression. A prime example is the *pim1*$^+$ gene (Matsumoto and Beach, 1991), whose mammalian homologue RCC1 encodes a protein that prevents premature initiation of mitosis but also participates in nuclear RNA processes (Dasso, 1993; Rush *et al.*, 1996). Mutations in the *Saccharomyces cerevisiae* homologue of RCC1 cause global defects in nuclear structure, RNA synthesis, and transport (Aebi *et al.*, 1990). A specific relationship between pre-mRNA splicing and cell-cycle progression is illustrated by the *dbf* mutants of *S. cerevisiae*. The *DBF3* and *DBF5* loci were identified in a screen for DNA synthesis mutants blocked in S phase, but they were found to be allelic with *PRP8* and *PRP3*, two genes required for pre-mRNA splicing (Johnston and Thomas, 1982; Shea *et al.*, 1994). These and other emerging examples point to a connection between RNA metabolism and regulation of the cell-division cycle.

The *S. pombe cdc28*$^+$ gene was initially identified in a screen for *cdc* mutants and was found to be required for entry into mitosis (Nasmyth and Nurse, 1981). In an effort to define more fully the requirements for cell-cycle progression, the present study describes the isolation and characterization of the *cdc28*$^+$ gene. The unexpected results of this investigation support a role for pre-mRNA splicing in the regulation of the cell cycle.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

All *S. pombe* strains used in this study were derived from those originally described by Leupold (1970) and are listed in Table 1. *S. pombe* was grown in standard YEA, YE, and phorbol 12-myristate 13-acetate (PMA) media (Beach *et al.*, 1985) containing additional amino acids at 75 μg/ml. In some cases phloxin B was added to solid medium at 20 μg/ml as an indicator of cell viability. So that the growth rate and viability of the *cdc28-P8* strain could be determined, mutant and wild-type cells were grown at 25°C in rich media (YEA) to mid-log phase and then shifted to the restrictive temperature (37°C). Cells were counted and replated on YEA at 25°C at the start of the shift to 37°C and at hourly intervals thereafter. Relative viability was calculated as the number of colonies at a given time point compared with the number at the start of the shift to 37°C. For fluorescence-activated cell-sorting (FACS) analysis, cells were grown in PMA containing 0.6 mM NH_4Cl for 48 h to arrest the cells in G1 and then inoculated into YEA at 37°C. Cells were removed at 2-h intervals and prepared as previously described (Costello *et al.*, 1986). For 4,6-diamidino-2-phenylindole (DAPI) staining, 10^7 cells grown to mid-log phase in liquid culture were washed in 5 ml of cold, deionized water. The cells were fixed in 1 ml of 2.5% glutaraldehyde for 10 min on ice and then washed twice in 1 ml of cold water. After resuspension in 25 μl of cold water, a mixture of 5 μl of cells and 5 μl of 75 μg/ml DAPI was prepared. A 2-μl sample was placed on a slide and observed with an Olympus PM-10AK fluorescence microscope with a DAPO 100 UV objective.

Cloning of cdc28$^+$

Standard molecular-cloning procedures were used (Sambrook *et al.*, 1989). We transformed (Ito *et al.*, 1983) SP968 with a genomic library (Caligiuri and Beach, 1993) constructed in pWH5 (Wright *et al.*, 1986). Transformants of leu$^+$ (3.2×10^4) were obtained at 25°C. The transformants were replica plated to YEA containing phloxin B and grown at 37°C to select for temperature-resistant (ts$^+$) clones. Eleven ts$^+$ transformants were tested for co-stability of the leucine prototrophy (leu$^+$) and the ability to grow at 37°C. Of the plasmids recovered from the ts$^+$ transformants (Beach *et al.*, 1982), four were able to complement the growth defect when reintroduced into SP968. Restriction analysis of these plasmids revealed that all four carried the same genomic fragment. The 7.5-kb insert from plasmid pcdc28-1 was subcloned, and the complementing activity was retained on a 4.8-kb *Bam*HI–*Bgl*II fragment that contained part of the pWH5 vector. Overlapping deletions of this fragment were constructed (Henikoff, 1987), and single-stranded DNA was prepared (Vieira and Messing, 1987). The sequence of both strands was determined with a 373A-automated sequencer (Applied Biosystems, Foster City, CA). An open reading frame (ORF) of ~3 kb was found, and the predicted amino acid sequence was compared with other proteins in the database by using the BLAST program (Altschul *et al.*, 1990). The complementing activity was shown to carry the *cdc28*$^+$ gene and not a multicopy suppressor by integration mapping. The *ars* sequence was excised from pcdc28-1 by digestion with *Eco*RI. A leu$^-$ strain was transformed to leucine prototrophy with

Table 1. *S. pombe* strains

972	h^{-S}
SP793	h^{-S} *cdc28-P8*
SP968	h^{+N} *cdc28-P8 leu1-32*
JP23	h^{-S} *prp1-1 ade6-216*
DK633	h^{-S} *prp5-1 leu1-32*
SU50-5B	h^{-S} *prp2-2*
SU26-9B	h^{-S} *prp8-1*

the plasmid, and stable transformants, because of chromosomal integration, were isolated. One of these integrants was crossed to a *cdc28-P8 leu1-32* strain to determine whether integration had occurred at the *cdc28* locus. Twenty-one tetrads were dissected, and in each case the leu and ts markers segregated 2:2. All leu^+ segregants grew at 37°C, and all leu^- segregants were ts^-. We therefore conclude that the gene we isolated integrated at the *cdc28* locus and is the authentic $cdc28^+$ gene. For genomic mapping the *Mlu* I fragment of the $cdc28^+$ gene was labeled with ^{32}P by random priming and used to probe an *S. pombe* genomic cosmid library on filters (Mizukami *et al.*, 1993).

Isolation of prp8-1

The *S. pombe* pre-mRNA splicing mutant *prp8-1* was isolated from a bank of temperature-sensitive lethal strains made by ethyl methane sulfonate (EMS) mutagenesis. Initial screening of the bank for the mutants defective in pre-mRNA splicing was done by hybridization of Northern blots with probes complementary to U6 small nuclear RNA. The *S. pombe* gene for U6 RNA is highly expressed and has an intron similar in structure to nuclear pre-mRNA introns (Tani and Ohshima, 1989). Defects in pre-mRNA splicing in each candidate were then confirmed by Northern blot analysis with the use of a probe for the β-tubulin mRNA whose gene has five introns (Hiraoka et al., 1984). Of 120 ts^- strains screened, we identified three mutants defective in pre-mRNA splicing. Complementation analysis with those mutants and previously isolated *prp* mutants (Potashkin *et al.*, 1989; D. Kim and D. Frendewey, unpublished results) showed that one of the mutants we isolated belongs to a new complementation group designated as *prp8*.

Genetic Linkage between cdc28 and prp8

Strains carrying the *cdc28-P8* allele were crossed with strains carrying one of nine different ts^- pre-mRNA splicing alleles: *prp1-1*, *prp2-1* (Potashkin *et al.*, 1989); *prp3-2*, *prp4-2*, *prp5-1*, *prp6-1*, *prp7-1*, *prp9-1* (Kim and Frendewey, unpublished data), and *prp8-1* (S. Uemura and T. Tani, unpublished data). For complementation grouping, diploid zygotes were isolated on rich medium at 23°C and tested for viability at 37°C. For linkage analysis, zygotes produced by the crosses were sporulated on maltose extract plates at 23°C. Unmated cells were killed and spores released by treatment with Glusulase (DuPont, Wilmington, DE). Random spores (800–2000) were germinated on a rich medium at 23 and 37°C, and the percentage of viable spores that grew at 37°C was determined.

Northern Analysis

Cultures were grown in rich media at 25°C to mid-log phase. Aliquots of the cultures were removed (time 0), and the remainder was shifted to 37°C. Samples were collected at 2, 4, 6, and 8 h after the temperature shift. Cell pellets were washed in RNase-free water and frozen at −70°C. The cells were lysed by vortexing with glass beads in 1 ml of solution A (0.32 M sucrose, 20 mM Tris-HCl, pH 7.5, and 10 mM EDTA) containing 0.5 mg/ml heparin. After centrifugation to remove the glass beads, the supernatant was transferred to a new tube and brought to 8 ml with solution B (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, and 1% SDS). The lysate was extracted twice with phenol and once with phenol/chloroform and ethanol precipitated twice. For analysis of pre-U6 RNA splicing, 10 μg of RNA from each time point was fractionated by electrophoresis on a 10% polyacrylamide/7 M urea gel. The RNA was transferred to Hybond (Amersham, Arlington Heights, IL) and fixed according to the manufacturer's instructions. The blot was hybridized with ^{32}P-labeled I-2 and E2-2 oligodeoxynucleotide probes against intron and second exon sequences of the U6 RNA precursor, as previously described (Potashkin *et al.*, 1989). For analysis of pre-mRNA splicing, 20 μg of total RNA was electrophoresed on a 1% formaldehyde agarose gel (Sambrook *et al.*, 1989), blotted onto a Gene Screen membrane (DuPont-NEN, Boston, MA) by the capillary method, and fixed by UV irradiation. The membrane was probed with a mixture of two ^{32}P-labeled oligonucleotide probes (TFII-IN1 and TFII-EX3) complementary to the first intron or the third exon of the *S. pombe* transcription factor IID (TFIID) pre-mRNA (Hoffmann *et al.*, 1990) in a solution containing 6× SSC, 50 mM sodium phosphate (pH 7.5), 2 mM EDTA, 0.1% SDS, 5× Denhardt's solution, and 0.2 mg/ml of preboiled salmon sperm DNA at 42°C. After hybridization, the membrane was washed three times in 6× SSC at room temperature and then once in 6× SSC and 0.1% SDS at 50°C for 5 min. Autoradiography was performed at −70°C with an intensifying screen for 2 d. Nucleotide sequences of the oligodeoxynucleotide probes for TFIID mRNA are as follows: TFII-IN1, 5′-GAAATCTCGTGACATGGTAG-3′; and TFII-EX3, 5′-GAGCTTGGAGTCATCCTCGG-3′.

RESULTS

The cdc28-P8 Mutation Causes Cell-Cycle Arrest in G2

Growth analysis of the *cdc28-P8* mutant indicated that it doubled once and then arrested upon shift to the restrictive temperature of 37°C (Figure 1A), and the cells became inviable (Figure 1B). Fluorescence-activated cell sorter (FACS) analysis demonstrated that *cdc28-P8* mutant cells grown in rich media accumulate in G2 when shifted to 37°C. In addition, we found that when *cdc28-P8* cells previously arrested in G1 by starvation for nitrogen were released into rich media, they arrested with a 2C-DNA content (Figure 1C, panel d). Because the fission-yeast cell cycle has a relatively long G2, most of the wild-type cells were also found to be in G2 (Figure 1C, panel c). These results demonstrate that *cdc28-P8* is able to proceed through G1 and S phases before arresting in G2, confirming the original observation of Nasmyth and Nurse (1981) that the $cdc28^+$ gene product executes its function in G2. To characterize the phenotype of the arrested cells, *cdc28-P8* was examined cytologically by staining with DAPI. Wild-type and mutant cells were grown at 25°C and then shifted to 37°C. Cells were collected at 0, 4, and 8 h after the shift to 37°C (Figure 1D). After 4 h at the restrictive temperature, the mutant cells (Figure 1D, panel b) were elongated and began to show cell-cycle arrest; after 8 h at 37°C, the cells were more elongated, and the nuclear material seemed to be condensed (Figure 1D, panel c). Wild-type cells at 37°C maintained the normal cellular and nuclear morphology (Figure 1D, panel d).

The $cdc28^+$ Gene Encodes an RNA-dependent ATPase/Helicase

We cloned the $cdc28^+$ gene by complementation of the temperature-sensitive (ts^-) growth impairment of *cdc28-P8*. A partial *Hin*dIII–*S. pombe* gene bank (Caligiuri and Beach, 1993) on a replicating plasmid carrying the *S. cerevisiae LEU2* gene, which complements *leu1-32*, was introduced into SP968 (*cdc28-P8 leu1-32*). We selected leu^+ transformants that grew at 37°C and

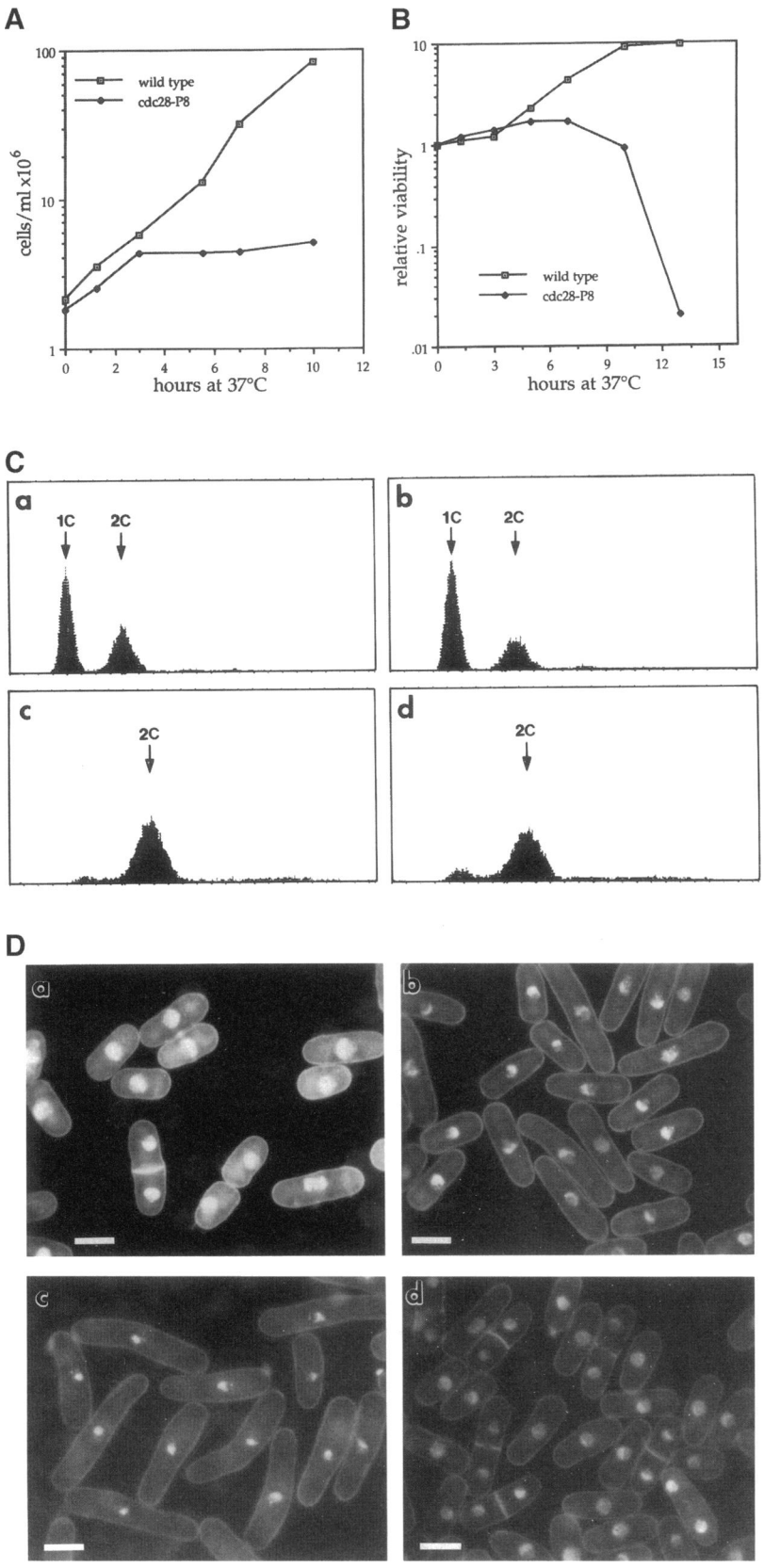


Figure 1. Phenotype of the *cdc28-P8* mutant. (A) Growth rates of *cdc28-P8* compared with the wild-type strain. Cells from cultures of exponentially growing *cdc28-P8* (SP793) and wild type (972) were counted at intervals after shifting to 37°C. (B) Cell viability. The relative viability of *cdc28-P8* and wild-type cells after shift to the restrictive temperature was determined by the ability to form colonies at 25°C. (C) FACS analysis. *cdc28-P8* and wild-type cells were grown in minimal media with limiting nitrogen at 25°C to starve the cells and arrest them in G1. The cells were then released into rich media at 37°C. Samples were removed for FACS analysis before and at 4 h after the temperature shift. Panels: (a) Wild type before the shift; (b) *cdc28-P8* before the shift; (c) wild type after 4 h at 37°C; and (d) *cdc28-P8* after 4 h at 37°C. (D) Fluorescence microscopy of DAPI-stained cells. Panels: (a) *cdc28-P8* at 25°C; (b) *cdc28-P8* after 4 h at 37°C; (c) *cdc28-P8* after 8 h at 37°C; and (d) wild-type cells at 37°C. Size bar, 5 μM.

tested them for co-instability of leucine prototrophy and temperature-resistant (ts^+) growth. Plasmids from four ts^+ transformants were able to complement the ts^- growth defect when reintroduced into SP968. Restriction analysis of these plasmids showed that all four contained identical *Hin*dIII fragments with a total insert size of 7.5 kbp. One of these plasmids (pcdc28-1) was chosen for further analysis (Figure 2). Integration mapping confirmed that the isolated DNA fragment is the *cdc28*$^+$ gene. Genomic mapping by hybridization to a cosmid library (Mizukami *et al.*, 1993) showed that *cdc28* lies between *cen2* and *ran1/pat1* on cosmid 1534 on the long arm of chromosome II.

A 4.8-kb *Bam*HI–*Bgl*II fragment (containing ~1 kb of pWH5 vector; Figure 2) retained the ability to complement the *cdc28-P8* mutation. We subjected this fragment to sequence analysis (Figure 3). The sequence contained an ORF of 2.9 kb that has the potential to encode a polypeptide of 968 amino acids with a predicted molecular mass of 111,500 Da. A comparison of this amino acid sequence with those in the databases by the BLAST program (Altschul *et al.*, 1990) revealed a similarity to a group of putative RNA-dependent ATPases or RNA helicases (Figures 3 and 4). The closest matches to the *cdc28*$^+$ ORF were the Prp2, Prp16, and Prp22 proteins from *S. cerevisiae* (Burgess *et al.*, 1990; Chen and Lin, 1990; Company *et al.*, 1991). The *cdc28*$^+$ gene product shares 56% identity with Prp2p and 50% identity with Prp22p and Prp16p in the central to carboxy-terminal region (580 amino acids) of each protein, which includes the DEAH box. The product of the *S. pombe prh1*$^+$ gene (Inoue *et al.*, 1992) also shares 43% identity with Cdc28 in this region (Figure 4).

The cdc28-P8 Strain Exhibits a Splicing Defect

The Prp2, Prp16, and Prp22 proteins each perform a distinct ATP-dependent step in pre-mRNA splicing (Company *et al.*, 1991; Schwer and Guthrie, 1991; Kim and Lin, 1993). We therefore tested for a general splicing defect in *cdc28-P8* by assaying the splicing of the U6 RNA precursor (pre-U6 RNA). U6 RNA is one of the spliceosomal small nuclear RNAs (snRNAs). The *S. pombe* U6 gene is interrupted by an intron whose structure is typical of nuclear pre-mRNA introns (Tani and Ohshima, 1989). In *S. pombe* pre-mRNA splicing (*prp*) mutants, the pattern of unspliced pre-U6 RNA accumulation is identical to that of pre-mRNAs (Potashkin and Frendewey, 1989). We compared the efficiency of pre-U6 RNA splicing in *cdc28-P8* with the wild type and with two *S. pombe prp* mutants: *prp1-1* (Potashkin *et al.*, 1989) and *prp5-1* (Kim and Frendewey, unpublished data). Total RNA was isolated from wild-type (972), *cdc28-P8* (SP968), *prp1-1* (JP23), and *prp5-1* (DK633) strains at 25°C and at 2, 6, and 8 h after the cells were shifted to 37°C. RNA from each strain was analyzed by Northern blotting (Figure 5). Two oligonucleotide probes that recognize the intron or second exon of pre-U6 RNA were used for hybridization.

Figure 5A shows the results of hybridization with the intron probe. The blot was autoradiographed for an extended period of time to allow for detection of rare or unstable intermediates or products of the splicing reaction (discussed below). Under these conditions, a band corresponding to the unspliced pre-U6 RNA was observed in nearly every lane. Essentially equal amounts of unspliced pre-U6 RNA accumulated in the wild-type and mutant strains at 25°C. The detection of U6 precursor at the permissive temperature was consistent with previous results (Potashkin and Frendewey, 1989), which indicated that the efficiency of splicing is reduced at lower temperatures. After shifting to 37°C, the pre-U6 RNA gradually disappeared in the wild type (972); none was detected 8 h after transfer to the restrictive temperature. In contrast, accumulation of unspliced precursor increased at 37°C in the splicing mutants and in *cdc28-P8* relative to the wild type and to the pre-U6 RNA levels at 25°C. A minor band visible below the pre-U6 RNA signal in the cdc28 RNA after 6 h at 37°C could suggest accumulation of a splicing intermediate, but it was not produced in sufficient amounts for identification. Other faint bands seem to be nonspecific background, as they are variable and do not correlate with strain or temperature differences. The accumulation of pre-U6 RNA in the mutants persists for at least 8 h at 37°C. These results were confirmed by hybridization with a probe against U6 exon-2. The autoradiogram shown in Figure 5B (a shorter exposure than in 5A) demonstrates that, like *prp1-1* and *prp5-1*, *cdc28-P8* accumulates pre-U6 RNA at the restrictive temperature, whereas the wild type does not. The highly stable, mature U6 RNA persists at the restrictive temperature even in severe mutants such as *prp1*, which has a complete block in splicing (Potashkin and Frendewey,

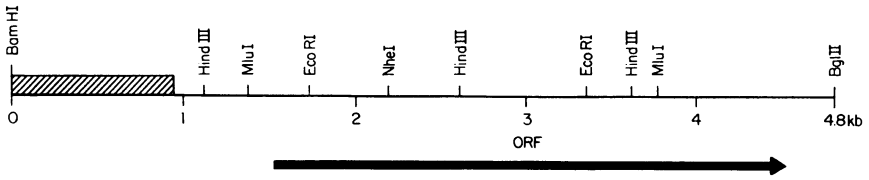
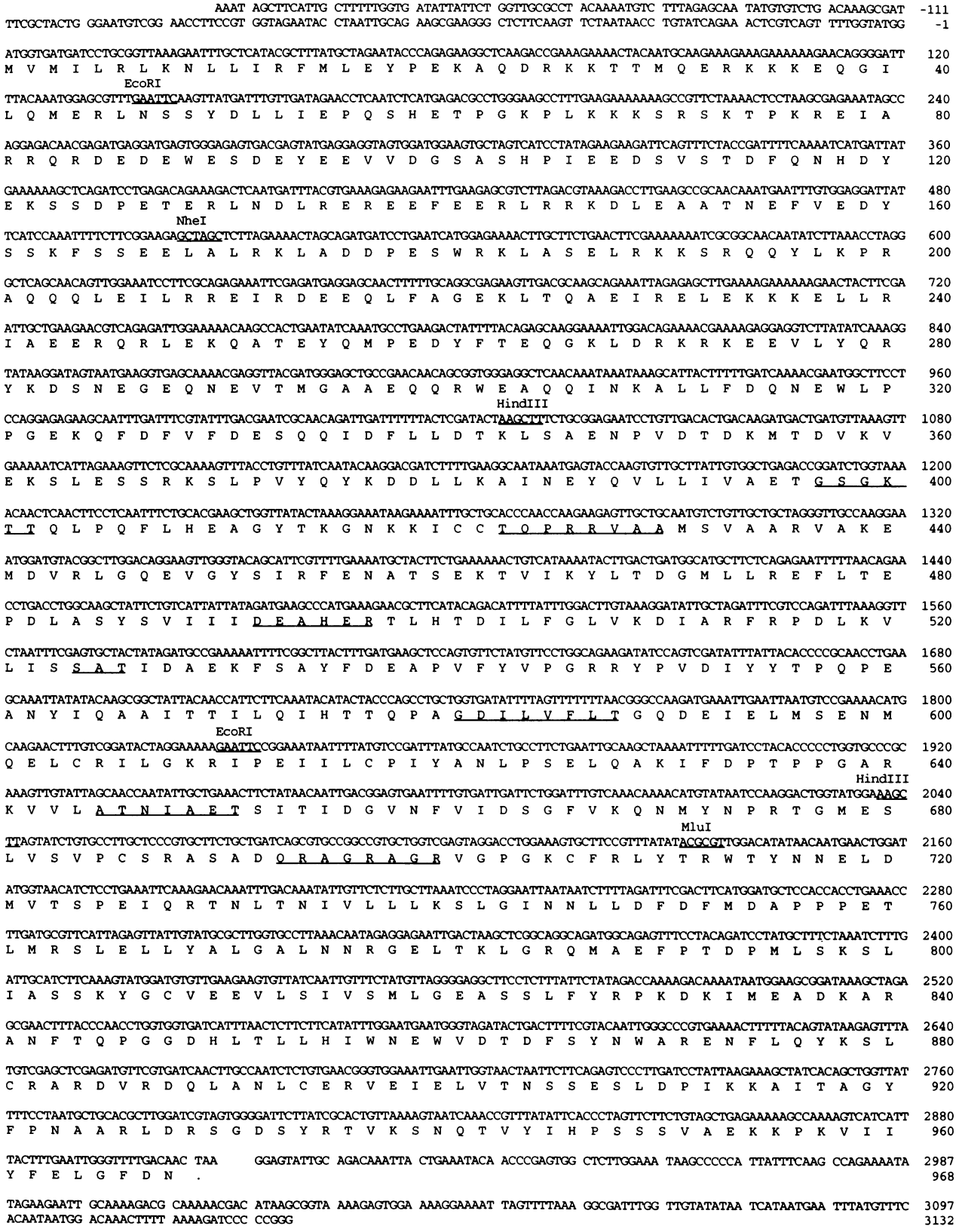


Figure 2. Restriction map of the 4.8-kb complementing fragment. The hatched area represents vector sequence; the arrow indicates the 2.9-kb open reading frame (ORF).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M V M I L R L K N L L I R F M L E Y P E K A Q D R K K T T M Q E R K K K E Q G I 40
EcoRI
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NheI
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HindIII
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L I S S A T I D A E K F S A Y F D E A P V F Y V P G R R Y P V D I Y Y T P Q P E 560
GCAAATTATATACAAGCGGCTATTACAACCATTCTTCAAATACATACTACCCAGCCTGCTGGTGATATTTTAGTTTTTTTAACGGGCCAAGATGAAATTGAATTAATGTCCGAAAACATG 1800
A N Y I Q A A I T T I L Q I H T T Q P A G D I L V F L T G Q D E I E L M S E N M 600
EcoRI
CAAGAACTTTGTCGGATACTAGGAAAAAGAATTCCGGAAATAATTTTATGTCCGATTTATGCCAATCTGCCTTCTGAATTGCAAGCTAAAATTTTTGATCCTACACCCCCTGGTGCCCGC 1920
Q E L C R I L G K R I P E I I L C P I Y A N L P S E L Q A K I F D P T P P G A R 640
HindIII
AAAGTTGTATTAGCAACCAATATTGCTGAAACTTCTATAACAATTGACGGAGTGAATTTTGTGATTGATTCTGGATTTGTCAAACAAAACATGTATAATCCAAGGACTGGTATGGAAAGC 2040
K V V L A T N I A E T S I T I D G V N F V I D S G F V K Q N M Y N P R T G M E S 680
MluI
TTAGTATCTGTGCCTTGCTCCCGTGCTTCTGCTGATCAGCGTGCCGGCCGTGCTGGTCGAGTAGGACCTGGAAAGTGCTTCCGTTTATATACGCGTTGGACATATAACAATGAACTGGAT 2160
L V S V P C S R A S A D Q R A G R A G R V G P G K C F R L Y T R W T Y N N E L D 720
ATGGTAACATCTCCTGAAATTCAAAGAACAAATTTGACAAATATTGTTCTCTTGCTTAAATCCCTAGGAATTAATAATCTTTTAGATTTCGACTTCATGGATGCTCCACCACCTGAAACC 2280
M V T S P E I Q R T N L T N I V L L L K S L G I N N L L D F D F M D A P P P E T 760
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L M R S L E L L Y A L G A L N N R G E L T K L G R Q M A E F P T D P M L S K S L 800
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I A S S K Y G C V E E V L S I V S M L G E A S S L F Y R P K D K I M E A D K A R 840
GCGAACTTTACCCAACCTGGTGGTGATCATTTAACTCTTCTTCATATTTGGAATGAATGGGTAGATACTGACTTTTCGTACAATTGGGCCCGTGAAAACTTTTTACAGTATAAGAGTTTA 2640
A N F T Q P G G D H L T L L H I W N E W V D T D F S Y N W A R E N F L Q Y K S L 880
TGTCGAGCTCGAGATGTTCGTGATCAACTTGCCAATCTCTGTGAACGGGTGGAAATTGAATTGGTAACTAATTCTTCAGAGTCCCTTGATCCTATTAAGAAAGCTATCACAGCTGGTTAT 2760
C R A R D V R D Q L A N L C E R V E I E L V T N S S E S L D P I K K A I T A G Y 920
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F P N A A R L D R S G D S Y R T V K S N Q T V Y I H P S S S V A E K K P K V I I 960
TACTTTGAATTGGGTTTTGACAAC TAA GGAGTATTGC AGACAAATTA CTGAAATACA ACCCGAGTGG CTCTTGGAAA TAAGCCCCCA TTATTTCAAG CCAGAAAATA 2987
Y F E L G F D N . 968
TAGAAGAATT GCAAAAGACG CAAAAACGAC ATAAGCGGTA AAAGAGTGGA AAAGGAAAAT TAGTTTTAAA GGCGATTTGG TTGTATATAA TCATAATGAA TTTATGTTTC 3097
ACAATAATGG ACAAACTTTT AAAAGATCCC CCGGG 3132

Figure 3.

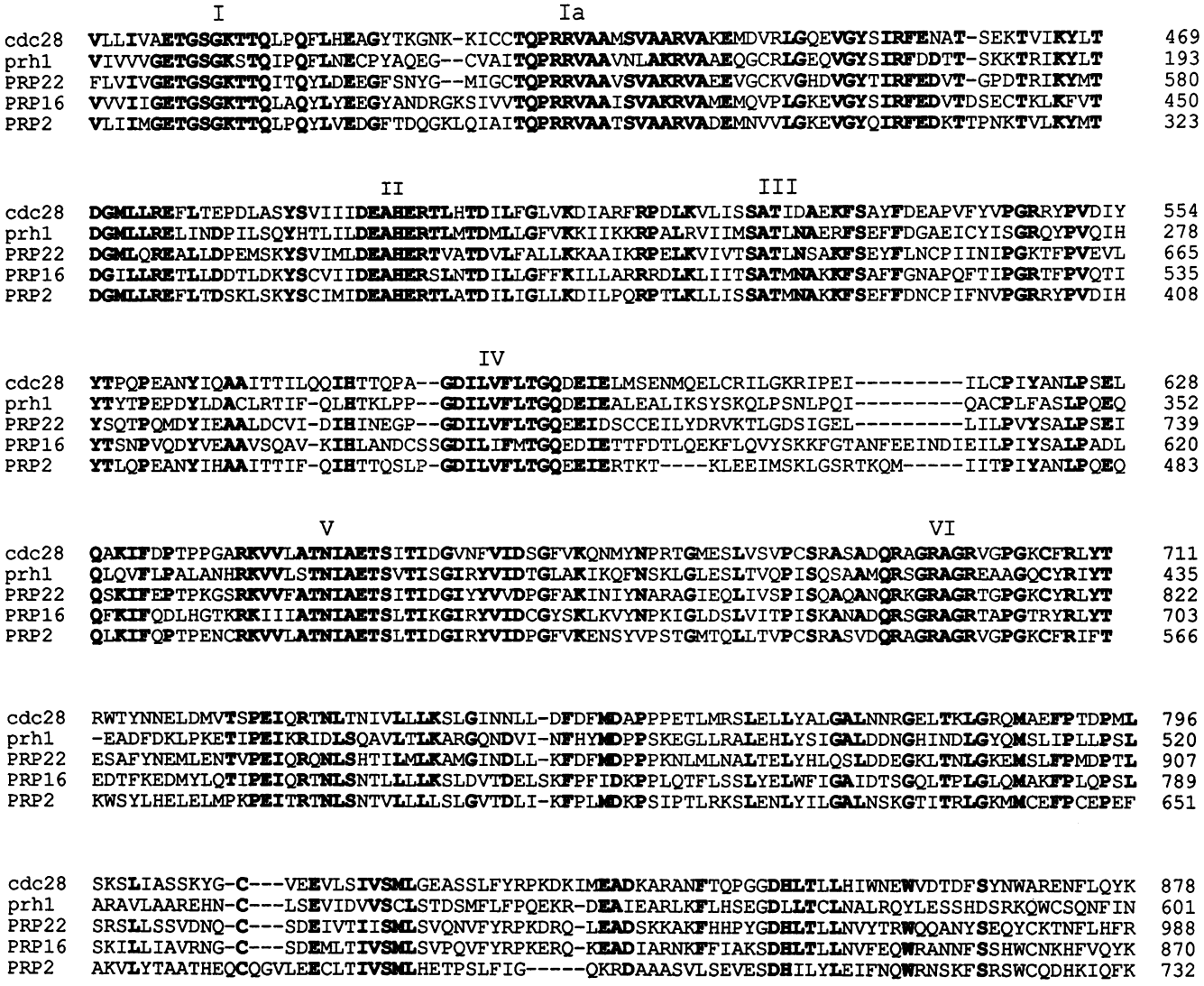

Figure 4. Alignment of the central region of the Cdc28 protein sequence with similar members of the DEAH-box RNA helicase family. The Prh1 protein is from *S. pombe,* and the Prp2, Prp16, and Prp22 proteins are from *S. cerevisiae.* Amino acids that are identical in all five or four of five of the proteins are emphasized in bold lettering. Roman numerals indicate the position of the motifs found in the DEAH-box putative RNA helicases (Company *et al.,* 1991).

1989). These results establish that cdc28-P8 exhibits a general defect in splicing roughly equivalent to that seen in two severe splicing mutants.

To confirm that *cdc28-P8* has a defect in pre-mRNA splicing as well as in pre-U6 RNA splicing, we performed Northern blot analysis with the use of probes for the TFIID mRNA. The *S. pombe* TFIID gene has three introns (Hoffmann *et al.,* 1990). As shown in Figure 6, the amount of the unspliced TFIID pre-mRNA greatly increases in *cdc28-P8* (lane 6), as it does in the two pre-mRNA splicing mutants *prp2-2* and *prp8-1* (lanes 4 and 8), after shifting to the restrictive temperature for 2 h (lane 6). In contrast, the wild-type cells do not accumulate pre-mRNA under the same conditions (lane 2). There is also a reduction in mature mRNA levels of the mutants at the restrictive temperature, which indicates a nearly complete block in splicing. These results establish that *cdc28-P8* exhibits a general defect in splicing roughly equivalent to that seen in four severe splicing mutants.

Figure 3 (cont). Nucleotide sequence of the 2904-bp ORF in the *cdc28*$^+$ gene and its predicted amino acid sequence (accession number U48733). No introns were detected. Restriction sites are indicated and underlined. The seven conserved regions for DEAH-box RNA helicases are underlined.

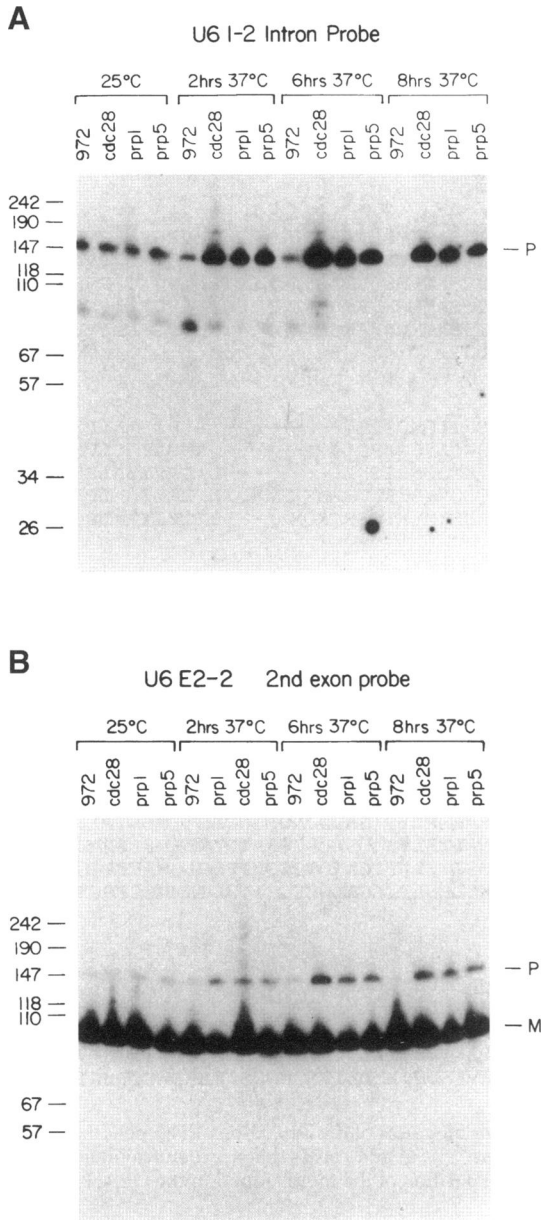

Figure 5. Northern blot analysis of pre-U6 RNA splicing. Wild-type (972) and mutant strains (*cdc28*, *prp1*, and *prp5*) were grown to mid-log phase and shifted to 37°C. Total RNA was isolated at 0, 2, 6, and 8 h after the temperature shift, analyzed on a 10% polyacrylamide/7 M urea gel, and transferred to Hybond membrane. The blot was probed with ^{32}P-labeled oligonucleotides U6 I-2 (A) and U6 E2-2 (B). Pre-RNA (P) is 149 nucleotides, and spliced, mature U6 RNA (M) is 99 nucleotides. The sizes in nucleotides of DNA markers are indicated on the left in each panel.

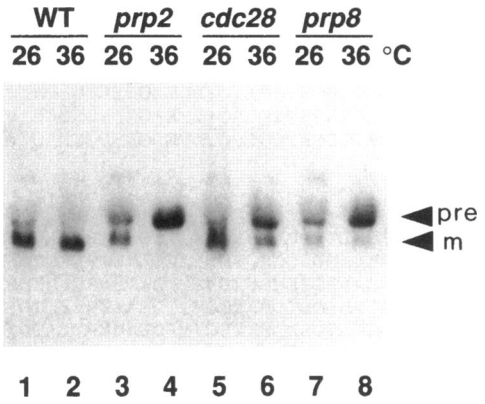

Figure 6. Northern blot analysis of pre-mRNA splicing. Wild-type (*972*) and mutant strains (*cdc28-P8*, *prp2-2*, and *prp8-1*) were grown to mid-log phase at 26°C and either maintained at 26°C or shifted to 36°C for 2 h. Total RNA was isolated from each strain and then subjected to Northern blot analysis. The blot was hybridized with a mixture of ^{32}P-labeled oligodeoxynucleotides TFII-IN1 and TFII-EX3, which are complementary to the first intron and the third exon of the pre-TFIID mRNA, respectively. The RNA source strains and the incubation temperatures are indicated above the lanes. WT represents the wild-type cells. Top and bottom arrowheads on the right indicate the positions of the unspliced precursor (pre) and mature (m) TFIID mRNAs.

cdc28 Is Allelic with prp8

Because *cdc28-P8* has a temperature-sensitive splicing defect, we wanted to determine whether *cdc28*$^+$ was identical to any of the genes identified by mutations that affect pre-mRNA splicing. Crosses between *cdc28-P8* and the previously described ts$^-$ alleles *prp1-1*, *prp2-1*, *prp3-1* (Potashkin *et al.*, 1989), and *prp4-1* Rosenberg *et al.*, 1991) or the new alleles *prp5-1*, *prp6-1*, *prp7-1*, and *prp9-1* (Kim and Frendewey, unpublished data) produced diploid progeny that were able to grow at 37°C. However, diploids produced from a cross between *cdc28-P8* and a new splicing mutant *prp8-1* remained temperature sensitive. Thus, *cdc28* and *prp8* are in the same complementation group. Genetic linkage analysis corroborated this finding. We found no temperature-resistant progeny among ~1400 random spores from a cross of *cdc28-P8* with *prp8-1*. From this tight linkage and the complementation grouping we conclude that *cdc28 and prp8* are identical loci.

cdc Phenotype of prp8-1

The *prp8-1* mutant divided twice before it ceased growing at the restrictive temperature. Like *cdc28-P8*, the cells began to elongate 6 h after the shift to the restrictive temperature. Almost all of the *prp8-1* cells elongated two- to threefold compared with the wild-type cells after 8 h at the restrictive temperature. Elongation of the cells at the restrictive temperature is a typical phenotype of the *cdc* mutants. Although the nuclear material in *cdc28-P8* seemed to be condensed after an 8-h shift, *prp8-1* did not show such a phenotype under the same conditions. Thus, chromatin condensation may be an allele-specific phenotype of *cdc28-P8*.

DISCUSSION

The *S. pombe cdc28-P8* strain was isolated in a screen for cdc mutants and was found to be blocked in late G2 or entry into mitosis at the restrictive temperature (Nasmyth and Nurse, 1981). We have confirmed the G2 arrest in *cdc28-P8* and showed that it is followed by a rapid decrease in cell viability. When we cloned the $cdc28^+$ gene by complementation of the temperature-sensitive growth defect in *cdc28-P8*, we were surprised to find that it encoded a protein of 111-kDa–predicted molecular mass that is similar to a family of putative RNA-dependent ATPases or helicases required for pre-mRNA splicing in budding yeast. Consistent with this similarity, *cdc28-P8* exhibits a block in the splicing of the pre-mRNA–type intron in the pre-U6 RNA (Tani and Ohshima, 1989) as well as in the splicing of pre-mRNAs at the restrictive temperature. An independent screen for *S. pombe* mutants defective in pre-mRNA splicing identified the *prp8-1* strain that, in addition to its splicing defect, also expresses a cdc phenotype seemingly identical to that of *cdc28-P8*. We demonstrated that *cdc28* and *prp8* are identical loci. Thus, a mutation in a single gene can cause a specific arrest in progression through the cell cycle and a block in pre-mRNA splicing.

The $cdc28^+/prp8^+$ gene product is most similar to the Prp2, Prp16, and Prp22 proteins of *S. cerevisiae*, which are members of the DEAH-box subfamily of putative RNA helicases. The product of the *S. pombe* $prh1^+$ gene (Inoue *et al.*, 1992), whose function is not known, is also a member of this family. Other possible members of the DEAH-box family have been identified in animal viruses (Koonin and Senkevich, 1992; Yanez *et al.*, 1993), humans (Ono *et al.*, 1994), budding yeast (James *et al.*, 1994), and *Escherichia coli* (Moriya *et al.*, 1995). The Cdc28/Prp8 protein is ~50% identical to Prp2p, Prp16p, and Prp22p in the most highly conserved C terminus. The eight characteristic motifs within the DEAD-box family of potential RNA helicases (Schmid and Linder, 1992) that are retained in the DEAH subfamily (Company *et al.*, 1991) are also present in Cdc28/Prp8, including the DEAH signature (amino acids 492–495), and TQPPRVAA (amino acids 423–430) and QRAGRAGR (amino acids 693–700), which are important for helicase activity (Company *et al.*, 1991; Schmid and Linder, 1992).

The Prp2, Prp16, and Prp22 proteins act at three sequential steps in pre-mRNA splicing. Prp2p is required early, just before the first transesterification reaction that results in cleavage at the 5′ splice site to produce the bimolecular intermediate, which consists of the upstream exon and the downstream exon attached to the intron in a lariat form (Lin *et al.*, 1987; King and Beggs, 1990; Kim and Lin, 1993). The second transesterification reaction that produces the spliced exons and releases the intron lariat requires Prp16p (Schwer and Guthrie, 1991). The final step in pre-mRNA splicing—the release of the spliced mRNA and intron lariat from the spliceosome—is promoted by Prp22p (Company *et al.*, 1991). These functions were first suggested by the molecules that accumulate in mutant strains: unspliced pre-mRNA in prp2, intermediates in prp16, and intron lariat in prp22. The *cdc28-P8* and *prp8-1* mutants accumulate unspliced precursors. Although a faint band was observed, splicing intermediates or final products were not observed to accumulate to significant levels in the mutants. This phenotype supports the notion that *S. pombe* Cdc28/Prp8 is homologous to *S. cerevisiae* Prp2p, but co-complementation experiments and further functional studies will be required to confirm this hypothesis.

The expression of both cdc and prp phenotypes in *cdc28-P8* and *prp8-1* joins a growing list of observations that point to a connection between RNA metabolism and regulation of the cell-division cycle. The earliest reported example of this phenomenon may be the peculiar observations that changes in tRNA expression can influence mitosis in fission yeast (Nurse and Thuriaux, 1984). Similar observations have recently been made in budding yeast (Reijo *et al.*, 1993). A better-understood example is the abundant mammalian nuclear protein RCC1, which prevents premature initiation of mitosis (Nishimoto *et al.*, 1978; Dasso, 1993). RCC1 is the guanine nucleotide exchange factor for the Ran GTPase, a central regulator of nuclear–cytoplasmic trafficking (Rush *et al.*, 1996). Mutations in the *S. cerevisiae* homologue of RCC1, *PRP20/SRM1*, have pleiotropic effects on nuclear structure and RNA metabolism, including accumulation of nuclear poly(A)$^+$ RNA and defects in pre-mRNA splicing (Aebi *et al.*, 1990). Two recent genetic results, whose meaning is at the moment obscure, support the notion of RNA-mediated events in the control of the cell cycle. In one case a *Drosophila* gene that encodes a DEAD-box RNA helicase can suppress loss of the key cell-cycle regulators Wee1 and Mik1 in *S. pombe* (Warbrick and Glover, 1994), and a gene that encodes a nuclear poly(A)$^+$ RNA binding protein (Wilson *et al.*, 1994) was isolated as a suppressor of a dominant mutation in the *S. cerevisiae CLN3* gene (Sugimoto *et al.*, 1995), which encodes a G1 cyclin.

More relevant to $cdc28^+$, the *dbf3-1* mutation in the *S. cerevisiae PRP8* gene, which encodes a protein of the U5 snRNP, produces a cell-cycle block before S phase (Shea *et al.*, 1994). Similar results were obtained with the budding-yeast mutants *dbf2* and *dbf5*, which also exhibit an S-phase arrest. *DBF5* was shown to be allelic with *PRP3* (Johnston and Thomas, 1982), and the *USS1* gene, which encodes a U6 snRNA binding protein, was isolated as a multicopy suppressor of *dbf2* (Cooper *et al.*, 1995). The *mis11-453* (Takahashi *et al.*, 1994) mutation, which results in minichromosome loss in *S. pombe*, was shown to reside in the $prp2^+$ gene, which

encodes a homologue of the large subunit of the human pre-mRNA splicing factor U2AF (Potashkin *et al.*, 1993). In a related connection between chromosome dynamics and pre-mRNA splicing, the *S. pombe dsk1*$^+$ gene, which encodes a kinase, was isolated as a multicopy suppressor of *dis1* mutants, which fail to separate sister chromatids at mitosis (Ohkura *et al.*, 1988; Takeuchi and Yanagida, 1993). The human homologue of Dsk1, SRPK1, causes nuclear disruption when overexpressed (Gui *et al.*, 1994). The SRPK1 kinase specifically phosphorylates members of the SR family of pre-mRNA splicing factors (Fu, 1995). The *dis1*$^+$ gene encodes a protein that associates with microtubules and the spindle pole bodies (Nabeshima *et al.*, 1995), a function that seems to have no connection with RNA processing. However, a conditional–lethal allele of *S. cerevisiae PRP22* elicits a loss of microtubules and a *cdc* arrest that produces large-budded cells at the restrictive temperature (T. Huffaker, personal communication). These disparate observations, along with the variety of cdc defects that have been observed in newly isolated *S. pombe* prp mutants (Kim and Frendewey, unpublished results; S. Urushiyama, T. Tani, and Y. Ohshima, unpublished results), suggest an intricate interplay between RNA-processing events and the regulation of the cell cycle.

What mechanisms could explain how mutations in splicing factors affect regulation of the cell cycle? A simple proposal is that a block in the splicing of the pre-mRNAs for key cell-cycle regulators leads to cell-cycle arrest at a point at which the regulator is required. This model does not, however, satisfactorily explain why only some of the known *prp* alleles in both yeast strains produce cdc phenotypes. All of the known prp genes in both *S. pombe* and *S. cerevisiae* seem to be required for constitutive splicing of all pre-mRNAs, yet different *prp* mutants elicit different cdc defects. These observations suggest a more global explanation that takes into account the role of the pre-mRNA splicing apparatus in nuclear structure. The nucleus contains a complex ribonucleoprotein network, and pre-mRNA splicing factors are localized to organized sites within this substructure (Spector, 1993). Several lines of evidence suggest that the spliceosome (Zeitlin *et al.*, 1989) or some of its components (Blencowe *et al.*, 1994; Zeng *et al.*, 1994; Chabot *et al.*, 1995) reside on the nuclear matrix. It is interesting with respect to cell-cycle regulation that the retinoblastoma protein associates with a nuclear matrix protein at subnuclear regions actively involved in RNA metabolism (Durfee *et al.*, 1994). The SR protein-splicing factors (Fu, 1995), in particular, seem to be tightly associated with nuclear matrix proteins (Blencowe *et al.*, 1994). These interactions between the splicing apparatus and the nuclear scaffold may need to be disrupted or reorganized to allow other nuclear events, such as mitosis and DNA replication, to proceed. The SRPK1/Dsk1 kinase (Ohkura *et al.*, 1988; Gui *et al.*, 1994) is a good candidate to promote this rearrangement of the ribonucleoprotein network of the nucleus. Likewise, RNA helicases, such as Prp22p from *S. cerevisiae* (Company *et al.*, 1991) and cdc28/prp8 from *S. pombe*, may facilitate disruption of the spliceosome as a prerequisite for nuclear division. Certain splicing factors could therefore serve dual roles as functional subunits of the spliceosome and as structural components of the nuclear matrix. Thus, mutations that affect pre-mRNA splicing could also prevent dynamic changes in nuclear structure required for cell-cycle progression. Some splicing factors, for example the helicases, could provide independent functions in mitotic processes such as spindle formation or chromosome separation. This hypothesis raises the intriguing possibility that RNA-mediated events could be crucial to the regulation of cell division.

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