Regulation of Cdc2p and Cdc13p Is Required for Cell Cycle Arrest Induced by Defective RNA Splicing in Fission Yeast*

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Screening of cdc mutants of fission yeast for those whose cell cycle arrest is independent of the DNA damage checkpoint identified the RNA splicing-deficient cdc28 mutant. A search for mutants of cdc28 cells that enter mitosis with unspliced RNA resulted in the identification of an orb5 point mutant. The orb5+ gene, which encodes a catalytic subunit of casein kinase II, was found to be required for cell cycle arrest in other mutants with defective RNA metabolism but not for operation of the DNA replication or DNA damage checkpoints. Loss of function of wee1+/or rad24+ also suppressed the arrest of several splicing mutants. Overexpression of the major B-type cyclin Cdc13p induced cdc28 cells to enter mitosis. The abundance of Cdc13p was reduced, and the phosphorylation of Cdc2p on tyrosine 15 was maintained in splicing-defective cells. These results suggest that regulation of Cdc13p and Cdc2p is required for G2 arrest in splicing mutants.

The sequence of cell cycle events is highly regulated to ensure the faithful duplication and segregation of the genome associated with cell division. Various surveillance mechanisms, or checkpoints, coordinate and monitor such events as DNA replication, DNA repair, spindle formation, reorganization of the actin cytoskeleton, and changes in cell size (1–4). If genotoxic stress results in damage to DNA, for example, checkpoint activation triggers cell cycle arrest before cells enter mitosis to provide sufficient time for the damage to be repaired. In many eukaryotes, the protein kinase Cdc2 (cyclin-dependent kinase 1) controls the onset of mitosis in a manner dependent on various internal and external conditions that include the presence of DNA damage, the status of DNA replication, nutrient availability, and cell size (5). The activity of Cdc2 is determined by the phosphorylation status of its Tyr15 residue and the availability of cyclin (5). Inhibitory phosphorylation of Cdc2 on Tyr15 is catalyzed by the tyrosine kinases Wee1 and Mik1, and the dephosphorylation of this residue is mediated predominantly by the tyrosine phosphatase Cdc25 (5).

Splicing of precursor mRNA (pre-mRNA) is essential for the expression of most protein-coding genes in eukaryotes and is mediated by the sequential assembly and rearrangement of small nuclear ribonucleoprotein complexes, or spliceosomes, on the pre-mRNA (6). After completion of the splicing reactions responsible for the excision of each intron, the spliceosome dissociates from the mature mRNA, and the excised introns are rapidly degraded. The prp (pre-mRNA processing) mutants prp5, prp6, prp8, prp11, prp12, prp13, prp14, and prp17 of fission yeast (Schizosaccharomyces pombe) manifest both accumulation of pre-mRNAs and the cell division cycle arrest (cdc) phenotype (7–10). In budding yeast (Saccharomyces cerevisiae), prp3, prp8, prp17, and prp22 mutants also show the cdc phenotype (11–14). Furthermore, DBF3 and DBF5 in budding yeast are required not only for DNA replication but also for pre-mRNA splicing (11). In mammalian cells, a component of a 40 S small nuclear ribonucleoprotein-containing complex that is a homolog of fission yeast Cdc5p and budding yeast Cef1 contributes to the G2-M transition of the cell cycle (15–17). A human Dim1 family protein is an evolutionarily conserved U5 small nuclear ribonucleoprotein protein, and its fission yeast ortholog is required for entry into mitosis (18, 19). Many genes are repressed during mitosis because of the down-regulation of transcription, polyadenylation of RNA, and translation (20), and pre-mRNA splicing has also been found to be targeted for inhibition during mitosis (21). These various observations suggest that regulation of a link between pre-mRNA splicing and the cell cycle is highly conserved. In addition, defects in degradation of the intron lariat result in a marked delay in cell cycle progression (22), further supporting the notion that defective RNA metabolism triggers cell cycle arrest.

Many cell cycle regulators have been identified in genetic screens for cdc mutants in fission yeast (5). Some cdc mutants, including cdc1 (subunit of DNA polymerase δ), cdc17 (DNA ligase), and cdc18 (homolog of budding yeast Cdc6), are defective in DNA metabolism, whereas others (cdc2, cdc13, or cdc25) are defective in the cell cycle machinery itself (5). In addition, two mutants, cdc5 and cdc28, have been found to be defective in pre-mRNA splicing. Whereas cdc5+ encodes a Myb-related protein, cdc28+ is allelic with prp8+ and encodes a DEAH box-containing an RNA helicase (9, 16).

Many cdc mutants with defects in DNA replication arrest in a manner dependent on the DNA damage checkpoint (5), whereas cdc mutants with defects in the cell cycle machinery arrest independently of this checkpoint (23). We have now screened for cdc mutants that arrest independently of the DNA damage checkpoint to reveal new mechanisms of cell cycle arrest. We found that pre-mRNA splicing-deficient cdc28 cells arrest independently of the DNA damage checkpoint, and we investigated the mechanism of cell cycle arrest in these cells.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Methods—Complete medium (YES, yeast extract plus supplement) and minimal medium (EMM, Edinburgh minimal medium) were prepared and standard methods were performed as described (24). The procedures for gene disruption and COOH-terminal tagging of proteins with HA or the Myc epitope were described previously (25). Transformation of S. pombe was performed by the lithium method (26). For microscopic analysis, cells were fixed with 70% ethanol and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described (27).

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The strains studied include HM4 (h− leu1-32 wt), HM202 (h− leu1-32 ura4-D18 cdc17-K42 [cdc17]), HM244 (h− leu1-32 ura4-D18 cdc18-K46 [cdc18]), HM520 (h− leu1-32 orp1-4 [orp1]), HM522 (h− orp1-1 we1-50 [orp1 we1]), HM560 (h− leu1-32 wee1-50 [wee1]), HM764 (h− leu1-32 ura4-D18 rad24::ura4+ [rad24]), HM1202 (h− leu1-32 cdc1-7 [cdc1]), HM1204 (h− leu1-32 swi7-H4 [swi7]), HM2855 (h− leu1-32 cdc28-P8 mik1::LEU2 we1-50 [cdc28 mik1 we1]), HM2863 (h− leu1-32 ura4-D18 cdc28-P8 rad24::ura4+ [rad24]), HM2892 (h− leu1-32 ura4-D18 wee1::HAhis-ura4+ [wt Wee1 HA], kindly provided by P. Russell), HM2895 (h− leu1-32 ura4-D18 wee1::HAhis-ura4+ cdc28-P8 [cdc28 Wee1-1 HA]), HM2905 (h− leu1-32 ura4-D18 cdc28-P8 tel1::kan rad3ts [cdc28 tel1 rad3]), HM2953 (h− prp13-1 [prp13]), HM2954 (h− prp11-1 [prp11], kindly provided by T. Tani). HM2031 (h− leu1-32 ura4-D18 cdc5-22 [cdc25]), HM3177 (h− leu1-32 cdc17-K42 orb5-3c13 [cdc17 orb5]), HM3178 (h− leu1-32 ura4-D18 cdc17-K42 we1-50 [cdc17 we1]), HM3180 (h− leu1-32 ura4-D18 cdc17-K42 rad24::ura4+ [cdc17 rad24]), HM3270 (h− cdc18-K46 orb5-3c13 [cdc18 orb5]), HM3271 (h− leu1-32 ura4-D18 cdc18-K46 rad24::ura4+ [cdc18 rad24]), HM3272 (h− leu1-32 ura4-D18 cdc18-K46 wee1-50 [cdc18 wee1]), HM3275 (h− cdc1-7 orb5-3c13 [cdc1 orb5]), HM3276 (h− leu1-32 ura4-D18 cdc1-7 rad24::ura4+ [rad24], cdc1 rad24), HM3277 (h− leu1-32 cdc1-7 wee1-50 [cdc1 we1]), HM3280 (h− leu1-32 ura4-D18 swi7-H4 orb5-3c13 [swi7 orb5]), HM3281 (h− leu1-32 ura4-D18 swi7-H4 wee1-50 [swi7 wee1]), HM3284 (h− leu1-32 ura4-D18 orb5-1-4 orb5-3c13 [orb5 orb5]), HM3321 (h− leu1-32 orb5-13myc:kan [wt orb5-Myc]), HM3325 (h− leu1-32 orb5-13myc:kan cdc28-P8 [cdc28 Orb5-Myc]), HM3399 (h− orb5-3c13 mik1::LEU2 cdc28-P8 leu1-32cdc8 orb5 mik1), HM3434 (h− leu1-32 orb5-3c13 [orb5]), HM3456 (h− cdc28-P8 cdc28-3HAkan [cdc28 cdc28]), HM3491 (h− leu1-32 ura4-D18 orb5-1-4 rad24::ura4+ [orb1 rad24]), HM3495 (h− leu1-32 wee1-50 mik1::LEU2 orb5-3c13 cdc28-P8 [cdc8 wee1 orb5 mik1]), HM3579 (h− leu1-32 cdc28-P8 wee1-50 [cdc28 wee1]), HM3588 (h− dbr1::kan wee1-50 [dbr1 wee1]), HM3589 (h− leu1-32 dbr1::kan [dbr1]), HM3605 (h− leu1-32 cdc28 orb5-3c13 [cdc28 orb5]), HM3612 (h− leu1-32 ura4-D18 dbr1::kan rad24::ura4+ [dbr1 rad24]), HM3621 (h− prp12-1 wee1-50 [prp12 wee1]), HM3630 (h− leu1-32 ura4-D18 prp12-1 rad24::ura4+ [prp12 rad24]), HM3667 (h− leu1-32 prp12-1 [prp12], kindly provided by T. Tani), HM668 (h− leu1-32 prp12-1 orb5-3c13 [prp12 orb5]), HM691 (h− leu1-32 cdc28-P8 [cdc28]), HM3821 (h− leu1-32 ura4-D18 ade6-M210 cdc5::cdc5 [cdc5]), kindly provided by K. Gould), HM3822 (h− leu1-32 cdc28-P8 orb5-3c13 wee1-50 [cdc28 orb5 wee1]), HM3836 (h− leu1-32 dbr1::kan orb5-3c13 [dbr1 orb5]), and HM3844 (h− leu1-32 orb5-3c13 wee1-50 [orb5 wee1]).

Isolation of Mutants with Abridged G2 Arrest in cdc28 Cells—cdc28 cells were mutagenized with 1-methyl-3-nitro-1-nitrosoguanidine (28, 29), spread on YES plates, and incubated for 4 days at 24 °C. Colonies (8 × 105) were replica-plated on YES medium containing PhloxinB (0.01 mg/ml) and incubated at 34 °C, and strains that grew poorly or died were collected. Cells that did not show the cdc phenotype were selected and crossed with the wt strain. Among those that were not linked to wee1−, we focused on one mutant designated 3c13. We transformed the double 3c13 cdc28 mutant with an S. pombe genomic library and found that orb5− suppressed the phenotype of 3c13 but not that of cdc28. We crossed the 3c13 mutant with the orb5-19 mutant, which was isolated as a morphology mutant defective in reestablishment of polarized growth (30). Among 5000 cells examined, there were no wt cells, indicating that orb5− is the gene affected by the 3c13 mutation. Indeed, we detected two point mutations in the open reading frame of orb5− in 3c13 cells; Glt− and Gly289 were changed to lysine and glutamic acid, respectively.

Protein Extraction and Immunoblot Analysis—Cell extracts were prepared and immunoblot analysis was performed as described (27). Extracts (0.06 mg of protein) were fractionated by SDS-polyacrylamide gel electrophoresis on a 10% gel, and the separated proteins were transferred to an Immobilon-P membrane (Millipore). Immunoblot analysis was performed with rabbit polyclonal antibodies to Cdc2p phosphorylated on Tyr15 (1:1000 dilution, Tyr15) as well as with mouse monoclonal antibodies to Cdc13p (1:1000 dilution; SP4, kindly provided by P. Nurse), to Cdc2p (1:1500 dilution, y63) to Myc (1:2000 dilution, Pharrmgen), to hemagglutinin (1:1000 dilution, Roche), and to α-tubulin (1:20000 dilution, Sigma). Immune complexes were detected with appropriate secondary antibodies (1:1000 dilution, Amersham Biosciences) and ECL reagents (Amersham Biosciences).

RESULTS

Cell Cycle Arrest Independent of the DNA Damage Checkpoint—To identify novel mechanisms of cell cycle arrest that are independent of the DNA damage checkpoint, we crossed several cdc mutants (cdc1, cdc2, cdc5, cdc10, cdc13, cdc17, cdc18, cdc19, cdc20, cdc21, cdc22, cdc23, cdc24, cdc25, cdc27, or cdc28) with the checkpoint-defective rad1 mutant (5). In the rad1 background, cdc2, cdc5, cdc10, cdc13, cdc25, and cdc28 mutants still manifested the cdc phenotype, whereas the other cdc mutants showed the “cut” phenotype, which is a marker for aberrant mitosis. Whereas cdc2−, cdc10−, cdc13−, and cdc25− encode important cell cycle regulators, cdc5− and cdc28− are required for pre-mRNA splicing (9, 16, 31). The mechanism of cell cycle arrest in these splicing mutants is largely unknown.

To confirm that the arrest of cdc28 cells is independent of the DNA damage checkpoint, we constructed a cdc28 rad3 te1 mutant. Most of these cells did not enter mitosis and arrested with the cdc phenotype at the restrictive temperature (Fig. 1a), verifying that cdc28 cells arrest independently of the DNA damage checkpoint.

Isolation of Mutants That Progress through Mitosis in the cdc28 Background—To clarify the mechanism of cell cycle arrest in cdc28 cells, we searched for mutants that progress through mitosis with defective pre-mRNA splicing. From 80,000 independent colonies, we isolated 10 mutants that failed to show the cdc phenotype and manifested reduced viability at the semirestrictive temperature. Genetic crosses and phenotypic analyses revealed that four of these mutants were linked to wee1−.
FIGURE 1. Cell cycle delay in cdc28 cells requires orb5\(^+\), wee1\(^+\), and rad24\(^+\). a–c, cells of the indicated genotypes were grown to late log phase, washed, and cultured in nitrogen-free medium for 14–15 h at 24 °C. They were then transferred to YES medium at time 0 and incubated at 36.5 °C for the indicated times, after which the proportion of cells with two nuclei was determined. At 6 h after the temperature shift, samples were also processed for staining with DAPI. Representative micrographs of the cells are shown in a and b. d, cells of the indicated genotypes were transformed with pcl-cdc2\(^+\), pcl-cdc2-F15 or the empty vector, grown to log phase at 24 °C. They were then transferred to 36.5 °C at time 0. After 8 h the proportion of the cut phenotype was determined. e, cells of the indicated genotypes were transformed with pcl-cdc13\(^+\) or the empty vector, grown to late log phase, washed, and cultured in nitrogen-free EMM medium for 14–15 h at 24 °C. They were then transferred to EMM medium containing nitrogen at time 0 and incubated at 36.5 °C for the indicated times, after which the proportion of binucleate cells was determined.
We next examined whether overexpression of cdc13+ induced cdc28 cells to enter mitosis (Fig. 1e). Although overexpression of cdc13+ did not affect the frequency of M phase entry in wt cells, it partially alleviated the arrest of cdc28 cells. These results suggest that the regulation of Cdc2p both by phosphorylation on Tyr15 and by Cdc13p contributes to the arrest of cdc28 cells.

orb5+, wee1+, and rad24+ Are Required for Cell Cycle Arrest in prp12 or dbr1 Cells—To test whether orb5+, wee1+, and rad24+ are required for cell cycle arrest in another pre-mRNA splicing-deficient mutant, we examined prp12 cells. Prp12p is required for formation of a functional U2 small nuclear ribonucleoprotein (7). We found that prp12 orb5 wee1, prp12 wee1, and prp12 rad24 cells entered mitosis, whereas most prp12 cells did not (Fig. 2a), suggesting that orb5+, wee1+, and rad24+ are all required for cell cycle arrest in prp12 cells.

We next investigated whether orb5+, wee1+, and rad24+ are required for cell cycle delay caused by a defect in RNA metabolism other than impaired pre-mRNA splicing. In fission yeast, dbr1 cells accumulate introns to high levels, grow slowly, and exhibit the cdc phenotype (22). Like the splicing mutants, dbr1 cells showed a marked delay in entry into mitosis, whereas dbr1 orb5, dbr1 wee1, or dbr1 rad24 cells entered mitosis more frequently (Fig. 2b). These results thus indicate both that orb5+, wee1+, and rad24+ are required for cell cycle delay in dbr1 cells as well as that accumulated introns induce cell cycle delay by a mechanism similar to that operative in splicing mutants.

Contribution of orb5+, wee1+, rad24+, and cdc13+ to Cell Survival in cdc28, prp12, or dbr1 Cells—If orb5+, wee1+, and rad24+ contribute to cell cycle arrest in splicing mutants, it would be expected that cell survival would decrease when the arrest is abrogated. To test this prediction, we determined the survival of G1-synchronized cells after shifting the incubation temperature to 36.5 °C for 8 h. The viability of cdc28 orb5, cdc28 wee1, or cdc28 rad24 cells decreased markedly as a result of incubation at the restrictive temperature, whereas that of the corresponding single mutants remained largely unchanged (Fig. 3a). Similarly, the viability of prp12 orb5, prp12 wee1, or prp12 rad24 mutants as well as that of dbr1 orb5, dbr1 wee1, or dbr1 rad24 cells was decreased by incubation at 36.5 °C, whereas that of prp12 and dbr1 single mutants remained constant. In addition, overexpression of cdc13+ also reduced the viability of cdc28 cells (Fig. 3b). These results suggest that the mech-

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* M. Shimada, C. Namikawa-Yamada, M. Nakanishi, and H. Murakami, unpublished data.
anism of cell cycle arrest regulated by orb5\(^+\), wee1\(^+\), rad24\(^+\), and cdc13\(^+\) is important for cell survival in RNA metabolism mutants.

orb5\(^+\) Is Not Required for the DNA Replication and DNA Damage Checkpoints—To investigate whether orb5\(^+\) is important for S phase arrest induced by inactivation of DNA replication proteins, we constructed double mutants of orb5 and several cdc mutants that arrest in S phase (cdc1, cdc17, cdc18, orp1, or swi7) (5, 28, 36). Orp1p is the largest subunit of the origin recognition complex, and Swi7p is the catalytic subunit of DNA polymerase. The cdc1 orb5, cdc17 orb5, cdc18 orb5, orp1 orb5, and swi7 orb5 mutants manifested the cdc phenotype similar to that of the corresponding single cdc mutants and most of the double mutant cells did not enter mitosis (Fig. 4, a and b), suggesting that orb5\(^+\) is not required for cell cycle arrest in the single cdc mutants. In contrast, a substantial proportion of cdc1 wee1, cdc17 wee1, cdc18 wee1, orp1 wee1, swi7 wee1, cdc1 rad24, cdc17 rad24, cdc18 rad24, and orp1 rad24 cells entered mitosis, suggesting that wee1\(^+\) and rad24\(^+\) contribute to cell cycle arrest in these mutants.

We next tested whether orb5\(^+\) is required for the arrest induced either by hydroxyurea, which inhibits DNA replication, or by methylmethane sulfonate, which induces DNA alkylation. Like wt cells, orb5\(^+\) cells showed the cdc phenotype after treatment with either of these agents (Fig. 4c). These data suggested that orb5\(^+\) is not required for operation of the DNA replication and DNA damage checkpoints.

cdc28 orb5, cdc28 wee1, and cdc28 rad24 Cells Progress through Mitosis with Unspliced mRNA—It was possible that cdc28 orb5, cdc28 wee1, and cdc28 rad24 cells entered mitosis by suppressing the splicing defect of cdc28 cells. To test this possibility, we performed Northern blot analysis of the tfId\(^+\) gene, which encodes a TATA box-binding factor and contains three introns (37). After incubation of cells for 4 or 8 h at 36.5 °C, substantial amounts of tfId\(^+\) pre-mRNA had accumulated in cdc28, cdc28 orb5, cdc28 wee1, and cdc28 rad24 cells but not in wt cells (Fig. 5). These results indicate that orb5, wee1, and rad24 do not suppress the splicing defect of cdc28 cells and that cdc28 orb5, cdc28 wee1, and cdc28 rad24 cells enter and progress through mitosis with unspliced mRNA.

orb5\(^+\), wee1\(^+\), and rad24\(^+\) Are Required Specifically for G2-M Arrest in cdc28 Cells—To confirm that orb5\(^+\), wee1\(^+\), and rad24\(^+\) are required specifically for G2-M arrest in cdc28 cells, we synchronized various mutants in early G2 phase by lactose gradient centrifugation. The temperature was shifted to 36.5 °C at time 0, and G2-M progression was halted by either hydroxyurea, which inhibits DNA replication, or by methylmethane sulfonate, which induces DNA alkylation. Like wt cells, orb5\(^+\) cells showed the cdc phenotype after treatment with either of these agents (Fig. 4c). These data suggested that orb5\(^+\) is not required for operation of the DNA replication and DNA damage checkpoints.
monitored (Fig. 6a). Whereas wt and orb5 cells entered mitosis at ~120 min after the temperature shift, wee1 cells did so much earlier (32). In contrast to cdc28 cells, cdc28 orb5 and cdc28 wee1 cells entered mitosis, although progression through mitosis was delayed in cdc28 orb5 cells. These results suggested that both orb5 and wee1 are required for G2 arrest in cdc28 cells. Similar to cdc28 wee1 cells, cdc28 rad24 cells entered mitosis earlier than did wt cells, suggesting that rad24 is also required for G2 arrest in cdc28 cells and that Rad24p is a rate-limiting negative regulator of the G2-M transition. However, it remains to be determined that Wee1, Orb5, and Rad24 are transducers or targets of the G2 arrest in splicing mutants.

**Phosphorylation of Cdc2p on Tyr15 and the Amount of Wee1p Are Maintained in cdc28 Cells—**Phosphorylation of Cdc2p on Tyr15 and the amounts of both the kinase Wee1p and the B-type cyclin Cdc13p are key determinants of the onset of mitosis (5, 23). We monitored the activities of the Tyr15-phosphorylated form of Cdc2p, total Cdc2p, Wee1p, Cdc13p, and Orb5p in wt and cdc28 cells synchronized by lactose gradient centrifugation (Fig. 6b). The abundance of Cdc2p remained virtually unchanged during the incubation of wt or cdc28 cells for up to 165 min. The amount of the Tyr15-phosphorylated form of Cdc2p decreased transiently during nuclear division in wt cells but was maintained at a high level in cdc28 cells. The level of Cdc13p gradually increased during nuclear division and then decreased in wt cells, whereas it maintained a...
The level of Cdc2p phosphorylation on Tyr15 remained unchanged in wt, cdc28, cdc28 orb5, and cdc28 orb5 mik1 cells, whereas it decreased in cdc28 wee1, cdc28 wee1 orb5, cdc28 mik1 wee1, and cdc28 mik1 wee1 orb5 cells (Fig. 7). The amount of the Tyr15-phosphorylated form of Cdc2p in cdc28 wee1 mik1 cells was lower than that in cdc28 wee1 cells. Similarly, the level of Cdc2p phosphorylation on Tyr15 in cdc28 mik1 wee1 orb5 cells was lower than that in cdc28 wee1 orb5 cells. Furthermore, the amounts of Tyr15-phosphorylated Cdc2p were similar in cdc28 wee1 orb5 cells and cdc28 wee1 cells as well as in cdc28 wee1 mik1 orb5 cells and cdc28 wee1 mik1 cells. These results suggest that wee1+ and mik1+, but not orb5+, have overlapping functions in the phosphorylation of Cdc2p on Tyr15.

Regulation of Cdc13p at the Protein and mRNA Levels in RNA Metabolism Mutants—Given that either overexpression of cdc13+ induced cdc28 cells to enter mitosis, we monitored the amounts of Cdc13p in several splicing-defective mutants after a temperature shift to 36.5 °C. The abundance of Cdc13p decreased in cdc5, prp12, and prp13 cells but not in cdc25 and swi7 cells (Fig. 8a). The reduced level of Cdc13p in the mutant cells was not simply attributable to cell cycle arrest, given that the amount of this protein was maintained at a high level in G2-arrested cdc5 cells and S-phase-arrested swi7 cells. Similarly, prp11 and db1 cells, but not wt cells, manifested down-regulation of Cdc13p in response to a temperature shift to 18 °C (Fig. 8b). The low abundance of Cdc13p in cdc28 cells appeared to be attributable, at least in part, to a low level of cdc13+ mRNA whose mobility was retarded at 4 and 8 h (Fig. 8c). However, the amount of cdc13+ mRNA was also low in cdc28 orb5, cdc28 wee1, and cdc28 rad24 cells, suggesting that maintenance of the Cdc13p level in cdc28 orb5 cells is not attributable to the up-regulation of cdc13+ mRNA.

**DISCUSSION**

Our investigation into the mechanism of cell cycle arrest in pre-mRNA splicing mutants of fission yeast has revealed the following. 1) These mutant cells arrest in a manner dependent on Cdc2p but not on proteins that mediate the DNA damage checkpoint, consistent with previous observations with cdc5 cells (16). 2) The arrest correlates with the maintenance both of Cdc2p phosphorylation on Tyr15 and of a low abundance of the B-type cyclin Cdc13p. 3) Wee1p, Rad24p, and Orb5p
are required for maintenance of the viability of the splicing mutants. 4) The reduced levels of Cdc13p in cdc28 cells are attributable at least in part to down-regulation of mRNAs. 5) Orb5p is required for cell cycle arrest in the splicing mutants but not for the DNA replication or DNA damage checkpoints.

There are at least two possible mechanisms by which defects in pre-mRNA splicing might halt cell cycle progression. One possibility is that proteins essential for entry of cells into mitosis are not expressed as a result of the splicing defect; 43% of the genes in fission yeast contain introns (38). At least three proteins are essential for mitotic entry: Cdc2p, Cdc13p, and Cdc25p. Cdc2p appeared to be expressed normally in the cdc28 mutant, even though the cdc25+ gene contains introns. In contrast, the amount of Cdc13p was decreased in the mutant, probably because of the low level of the corresponding mRNA, even though the cdc13+ gene does not contain introns. Similarly, the amounts of both Cdc25p and cdc25+ mRNA did not vary significantly in cdc28 cells, again even though the cdc25+ gene does not contain introns. The reduced expression of Cdc13p in the mutant cells is thus not directly attributable to defective pre-mRNA splicing. Rather, transcription of cdc13+ gene or degradation of the mature mRNAs is likely to be affected indirectly by the splicing defect. In addition, the slower migrating form of cdc13+ mRNA was observed in the cdc28 mutant. The reason for this slow migration is unknown at present, but it is possible that pre-mRNA containing no intron is also processed by the splicing machinery, which may affect transcription initiation site or polyadenylation. However, we cannot exclude the possibility that the mechanism of G2 arrest in splicing mutants might be because of the reduced expression of other genes essential for G2-M progression. Screening for those genes that suppress G2 arrest of splicing mutants using cDNA library would help the elucidation of this mechanism. The other possible explanation for the cell cycle arrest induced by defective pre-mRNA splicing is that a regulatory system such as the DNA damage checkpoint is responsible. It is likely that such a checkpoint-like system operates in fission yeast, given that cdc28 cells with orb5, wee1, or rad24 mutations were found to enter mitosis with unspliced pre-mRNA. In budding yeast, cell cycle arrest in cef1 cells, which are defective in pre-mRNA splicing, results from inefficient splicing of the TUB1 gene, which encodes the major isofrom of α-tubulin (39). Similar to cef1 cells, the cell cycle arrest apparent in other mutants of budding yeast with defects in pre-mRNA splicing is because of activation of the spindle checkpoint (40). Cell cycle arrest in cdc28 cells of fission yeast was not attributable to inefficient splicing of a tubulin gene, given that mutation of spindle checkpoint proteins (nud2 or nud1) or the expression of α-tubulin cDNA (nud2+ or nud3−) failed to suppress G2 arrest in these cells. The mechanisms of cell cycle arrest induced by defects in pre-mRNA splicing thus appear to differ between fission yeast and budding yeast.

In mammalian cells, expression of a dominant negative mutant of human Cdc5, a homolog of fission yeast Cdc5p, slowed G2 progression and delayed entry into mitosis, whereas overexpression of wild-type human Cdc5 shortened the G2 phase (15), suggesting that the link between cell cycle regulation and pre-mRNA splicing is conserved among eukaryotes. Consistent with this notion, cyclin E associates with components of the pre-mRNA splicing machinery in mammalian cells (41) and Cdc2p of mammalian cells phosphorylates the splicing factor SF2 (ASF) (42).

The mechanism of cell cycle arrest caused by defects in pre-mRNA splicing in fission yeast differs from that caused by inhibition of DNA replication or by DNA damage in several respects. First, proteins that mediate the DNA replication or DNA damage checkpoints are not required for the arrest because of deficient splicing; cdc28 rad3 tel1 cells thus failed to enter mitosis. Rad1p is also not required for the arrest in cdc5 cells (16). Second, Orb5p is required for cell cycle arrest in splicing mutants but not for the operation of the DNA replication or DNA damage checkpoints. Cell cycle progression in cdc28 orb5 cells was not identical to that in cdc28 wee1 cells. The frequency of the cut phenotype was low in the former cells, even though they progressed through mitosis with unspliced mRNA, probably because DNA replication was completed, and DNA was not damaged and because the orb5 mutation, unlike the wee1 mutation, did not advance cell cycle progression. Third, a level of Cdc13p is negatively correlated with cell cycle arrest in splicing mutants, given that overexpression of cdc13+ abrogated arrest, and Cdc13p is maintained at a low concentration in cdc28 cells. In contrast, the abundance of Cdc13p is not affected markedly and overexpression of cdc13+ does not induce mitosis in cells in which the DNA replication or DNA damage checkpoint is activated (43).

There are several common characteristics of cell cycle arrest induced by defective pre-mRNA splicing or by activation of the DNA replication or DNA damage checkpoints. The phosphorylation of Cdc2p on Tyr15 is required for arrest in all these instances (5). Furthermore, Rad24p is required for cell cycle arrest in response either to defective splicing or to DNA damage (1, 5). Budding yeast with a defect in synthesis of the cell wall also halts cell cycle progression at G2 (44). This cell wall checkpoint requires down-regulation of B-type cyclin, similar to that observed in the splicing mutants of fission yeast.

The Vpr protein of human immunodeficiency virus-type 1 induces G2 arrest in both human and fission yeast cells through phosphorylation of Cdc2p on Tyr15 (35, 45). This cell cycle arrest requires Wee1p and Rad24p but not proteins that mediate the DNA damage or replication checkpoints. The mechanism of Vpr-induced arrest is similar to that apparent in the splicing mutants. The proteins with which Vpr interacts, which remain to be identified, might thus also mediate cell cycle arrest in splicing mutants.

As is the case for the DNA replication and DNA damage checkpoints, abrogation of cell cycle arrest reduced the viability of splicing mutants. Similar to transcription, polyadenylation, and translation, splicing is a target for mitotic inhibition (20, 21). Such controls likely ensure that inappropriate gene expression does not occur during mitosis, thereby preventing aberrant proteins or RNAs from acting in a dominant negative manner to inhibit normal gene function. Progression of cells into M phase with unspliced RNA may therefore directly result in a loss of viability in splicing mutants. These facts imply that cells are equipped with the ability to arrest in G2 in the presence of unspliced RNA.

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