

On the Slowing of S Phase in Response to DNA Damage in Fission Yeast*

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Eukaryotic cells slow their progression through S phase upon DNA damage. The mechanism that leads to this slowing is called the intra-S-phase checkpoint. Previous studies demonstrated that in the fission yeast *Schizosaccharomyces pombe* this checkpoint is mediated by a pathway that includes Rad3 (similar to human ATR and ATM) and Cds1 (similar to human Chk1 and Chk2). Here we present evidence that a major downstream target of this pathway is the cyclin-dependent kinase, Cdk2. We also present evidence suggesting that the intra-S-phase checkpoint makes a relatively minor contribution to the survival of cells with damaged DNA.

Eukaryotic cells actively slow their progression through S phase when their DNA is damaged (for review, see Ref. 1). The biological importance of this response, which is called the intra-S-phase checkpoint, is not clear, but it is frequently assumed that this checkpoint provides cells with time to repair the damaged DNA before its replication. Interest in the intra-S-phase checkpoint is enhanced by the strong correlation in several human genetic diseases between loss of the intra-S-phase checkpoint and susceptibility to cancer (2).

In all tested eukaryotic organisms the intra-S-phase checkpoint depends on the function of one or two members of the ATR family of protein kinases (ATR and ATM in vertebrate cells (3–5), Mec1 and Tel1 in the budding yeast, *Saccharomyces cerevisiae* (6–8) and Rad3 and Tel1 in the fission yeast, *S. pombe* (9, 10)). The relative roles of the ATR-like kinases depend on the nature of the damage. For example, in vertebrate cells ATM is primarily responsible for the checkpoint response to damage by ionizing radiation (3). In contrast, ATR is the principal mediator of the checkpoint response to ultraviolet light damage and alkylation damage (5, 11).

Most if not all of the intra-S-phase checkpoint pathways downstream of the ATR family depend on one or another member of the Chk family of protein kinases (Chk1 and Chk2 in vertebrates, Chk1 and Rad53 in budding yeast, Chk1 and Cds1 in fission yeast). The Chk kinases become active when they are phosphorylated by an ATR-family kinase (3, 4).

Events downstream of the Chk kinases are best understood in vertebrate cells. One of the principal targets of the Chk kinases is the cyclin-dependent kinase, Cdk2, but Cdk2 is not

directly regulated by the Chk kinases. Instead, Cdk2 is inhibited by phosphorylation of its tyrosine 15 (Tyr-15) and activated by removal of the phosphate at Tyr-15. Cdc25A is the phosphatase that activates Cdk2. Phosphorylation of Cdc25A by Chk1 or Chk2 leads to its degradation. Thus, phosphorylated Cdc25A cannot dephosphorylate and activate Cdk2 (3, 12–14). Cdk2 (in combination with cyclins E or A) is required for initiation of DNA replication in vertebrates (15, 16). Thus inactivation of Cdk2 could slow S phase by reducing the rate of initiation at origins. Inhibition of Cdk2 may slow S phase by other mechanisms as well.

Rad53 is the Chk kinase that is important for the intra-S-phase checkpoint in budding yeast (7), and Cds1 is the relevant Chk kinase in fission yeast (9, 10). Whether DNA-damage-induced activation of Rad53 or Cds1 slows S phase by inhibiting a cyclin-dependent kinase or in some other way is not known.

Most previous studies of the intra-S-phase checkpoint in budding and fission yeast have employed the methylating agent, methyl methane sulfonate (MMS),¹ to introduce damage into DNA (7, 9, 10, 17, 18). In the case of fission yeast these studies have demonstrated that the MMS-induced intra-S-phase checkpoint depends on Rad3 (similar to vertebrate ATR), Rad26 (similar to vertebrate ATRIP), and the group of proteins that presumably loads a checkpoint-specific PCNA-like structure onto damaged DNA (Rad17, Rad9, Rad1, and Hus1; similar to the vertebrate proteins with the same names (9, 10)). The fission yeast intra-S-phase checkpoint also requires Rad4/Cut5 (10), which is similar to vertebrate TopBP1/Cut5, and presumably Swi1, which is similar to budding yeast Tof1 (19). These proteins appear to act cooperatively to facilitate the activation of Cds1 (similar to vertebrate Chk2 and budding yeast Rad53 but with some of the functions of vertebrate Chk1).

In budding yeast the MRX complex is also required for the intra-S-phase checkpoint (8). This complex consists of Mre11 (similar to Mre11 in vertebrates and Rad32 in fission yeast), Rad50 (similar to Rad50 in vertebrates and fission yeast), and Xrs2 (similar to Nbs1 in vertebrates and fission yeast). The corresponding complex in vertebrates and fission yeast is called the MRN complex, and it is likewise essential for the intra-S-phase checkpoint in these organisms (2, 18). The MRX and MRN complexes in budding yeast and vertebrates function upstream of Rad53 and Chk2, respectively (20, 21), but the relationship between the MRN complex and Cds1 in fission yeast has not yet been defined (18).

The pathways downstream of Cds1 for the intra-S-phase checkpoint in fission yeast have not previously been identified. We suspected that one of these pathways might resemble the

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¹ The abbreviations used are: MMS, methyl methane sulfonate; Cdk, cyclin-dependent kinase.

TABLE I
Yeast strains used in this study

Mutation	Strain name	Genotype	Source/Reference
None	501	<i>h- ura4-D18 leu1-32 ade6-704</i>	10
<i>cds1Δ</i>	SK02	<i>h- ura4-D18 leu1-32 ade6-704 cds1::kan-MX6</i>	This study
<i>cdc25OP</i>	TE387	<i>h- adh::cdc25+ cdc25::ura4 leu1-32 ura4-D18</i>	A gift from Tamar Enoch
<i>wee1-50 mik1Δ</i>	TE386	<i>h- wee1-50 mik1::LEU2 leu1-32</i>	A gift from Tamar Enoch
<i>mik1Δ</i>	SP975	<i>h+ ade6-M210 leu1-32 mik1::ura4 ura4-D18</i>	A gift from Nancy Walworth
<i>cdc2Y15F</i>	KGy14	<i>h- cdc2::ura4+ cdc2Y15F::LEU2 ura4-D18 leu1-32</i>	A gift from Kathy Gould
<i>rad3Δ</i>	1378	<i>h- rad3::ura4 ura4-D18 leu-32 ade6-704</i>	10
<i>wee1-50</i>	TK27	<i>h+ wee1-50 leu1-32 ura4-D18</i>	A gift from Grant Brown
<i>cdc10-v50</i>	SZ290	<i>h+ cdc10-v50 ura4-D18 leu1-32 ade6-M210</i>	A gift from Greg Freyer
<i>cdc10-v50 cdc25OP</i>	SK08	<i>h- cdc10-v50 cdc25::ura4+ ura4-D18 adh::cdc25+ ade?</i>	This study
<i>cdc10-v50 cdc2Y15F</i>	SK09	<i>h- cdc10-v50 cdc2::ura4+ cdc2Y15F::LEU2 ade?</i>	This study

vertebrate pathway discussed above. According to this hypothesis, checkpoint-induced activation of Cds1 would lead to phosphorylation of Cdc25 (the fission yeast homolog of vertebrate Cdc25A), which in turn would lead to inhibition of Cdc2 (the fission yeast cyclin-dependent kinase) and, thus, to slowing of S phase. Support for this hypothesis came from our previous finding (10) that the upstream portions (from signal generation to activation of Cds1) of the MMS-induced intra-S-phase checkpoint in fission yeast appeared to be identical to the upstream portions of the S_{dNTP} -M checkpoint (which blocks entry into mitosis when cells are kept in S phase as a consequence of starvation for deoxynucleoside triphosphates by treatment with hydroxyurea). If the upstream portions of the S_{dNTP} -M checkpoint and the intra-S-phase checkpoint are identical, then might not the downstream portions (downstream of Cds1) also be similar or identical? It was already known that the downstream portion of the S_{dNTP} -M checkpoint is primarily a consequence of Cds1-mediated inactivation of Cdc25 (22). Inactivation of Cdc25 prevents activation of Cdc2 and, thus, prevents mitosis. Because Cdc2 is also required for initiation of DNA replication (23–25), it seemed plausible to us that both the S_{dNTP} -M checkpoint and the intra-S-phase checkpoint might be mediated by the inhibition of Cdc2.

For these reasons we have carried out a study in fission yeast to evaluate the potential roles of Cdc25 and Cdc2 in the intra-S-phase checkpoint. We also tested the potential roles of the Wee1 and Mik1 kinases, which are responsible for inhibiting Cdc2 by phosphorylating it on Tyr-15 (26, 27) and which also contribute to the S_{dNTP} -M checkpoint (28, 29). Our results support the hypothesis that a major portion of the intra-S-phase checkpoint in fission yeast is a consequence of phosphorylation-mediated inhibition of Cdc2, similar to the S_{dNTP} -M checkpoint in fission yeast and vertebrates and similar to the intra-S-phase checkpoint in vertebrates.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—Yeast strains used for this study are listed in Table I. All strains were cultured in YES medium at 30 °C (unless otherwise indicated) with vigorous shaking. YES consisted of 0.5% yeast extract (Difco), 3% glucose; histidine, uracil, and adenine were added to 75 mg/liter, whereas leucine was added to 200 mg/liter (all chemicals were from Sigma). For plates, 2% agar (Difco) was added to the medium. The strain labeled SK02 was made by replacing the *cds1+* gene with a Kan-MX6 cassette using the methodology described by Bahler *et al.* (30). SK08 and SK09 were made by crossing *cdc10-v50* with TE387 and KGy14, respectively.

Assay for the Intra-S-phase Checkpoint—For the log-phase method, cells were grown to 1×10^7 cells/ml overnight, diluted to 1×10^6 /ml, and allowed to recover for 1 h. Then the cultures were left untreated or treated with 0.0075 or 0.015% MMS (Sigma), and the 0-h samples were collected for flow cytometry by fixing a small aliquot of cells in 70% ethanol. Additional samples for flow cytometry were collected on an hourly basis. For the *cdc10* method, cells were grown to 1×10^7 cells/ml overnight at 25 °C, diluted to 5×10^6 cells/ml, and allowed to recover for 1 h at 25 °C. Cells were then incubated at 35 °C for 4 h to arrest them with a 1N DNA content. Cells were released from the block by lowering

the temperature to 25 °C. 0.015% MMS was added (or not) at the time of release, and samples were collected for flow cytometry at 30-min intervals. Flow cytometric analysis was carried out as described previously (10) except that FlowJo software (TreeStar Inc.) was used to generate the flow histograms.

MMS Sensitivity Assays—To measure sensitivity to MMS as a function of time, cells were grown at 25 °C to 1×10^7 cells/ml, diluted to 5×10^6 cells/ml, allowed to grow for another hour, treated with 0.015% MMS, and further incubated at 25 °C. Samples were collected every 2 h. For each time point three different numbers (50, 500, and 5000) of cells were plated onto separate YES-agar plates lacking MMS. The plates were incubated at 25 °C. For each time point the results for all three dilutions were mutually consistent. The surviving colonies on the plates with the largest numbers of resolvable colonies were counted, and the S.D. of the count (the square root of the number counted) was determined. These numbers were then expressed as percentages relative to the initial values at the time of addition of MMS.

To measure the ability to grow in the presence of MMS, cells were grown to log phase overnight, counted, and diluted to 1×10^7 cells/ml. 10-Fold serial dilutions were made, and 5 μ l of each dilution were spotted onto YES agar plates containing 0.005% or 0.010% MMS. The plates were incubated at 25 °C for 4 days, then photographed.

RESULTS

A New Method to Evaluate the Intra-S-phase Checkpoint—We previously used two cell synchronization methods ((i) nitrogen starvation and release and (ii) temperature block and release of *cdc25* temperature-sensitive mutant strains) to measure the MMS-induced intra-S-phase checkpoint (10). Nitrogen-starved *S. pombe* cells arrest with 1N DNA content in a G_0 -like state. When transferred to rich medium, the cells resume cycling, pass through S phase, and enter G_2 phase. However, cells emerge asynchronously from the G_0 block, which makes the nitrogen starvation/release method insensitive to partial phenotypes (10). The *cdc25* method produces better synchrony. At high temperatures cells bearing the *cdc25* mutation cannot enter mitosis, so the cells arrest in late G_2 phase. When the temperature is lowered, the cells pass through mitosis and the subsequent G_1 and S phases in good synchrony, thus permitting detection of partial intra-S-phase checkpoint phenotypes (10). However, in the present study we employed mutants that cannot be synchronized by *cdc25* block and release, because these mutants contained their own defects in the Cdc25 pathway. Furthermore, the mutants in the present study were also defective in G_0 arrest upon nitrogen starvation. Therefore, we developed an alternative method to measure checkpoint-dependent S-phase delay.

For this purpose we optimized a simple assay (suggested to us by Dr. Grant Brown at the University of Toronto) to investigate the intra-S-phase checkpoint in *S. pombe* cells. The assay involves growing *S. pombe* cells to log phase in rich medium and treating them or not with low concentrations of MMS, then collecting hourly samples for flow cytometry. In rich medium *S. pombe* cells have rapid G_1 and S phases, and they do not undergo cytokinesis until near the end of S phase. Most of the cell cycle is spent in G_2 phase. Consequently, the majority of

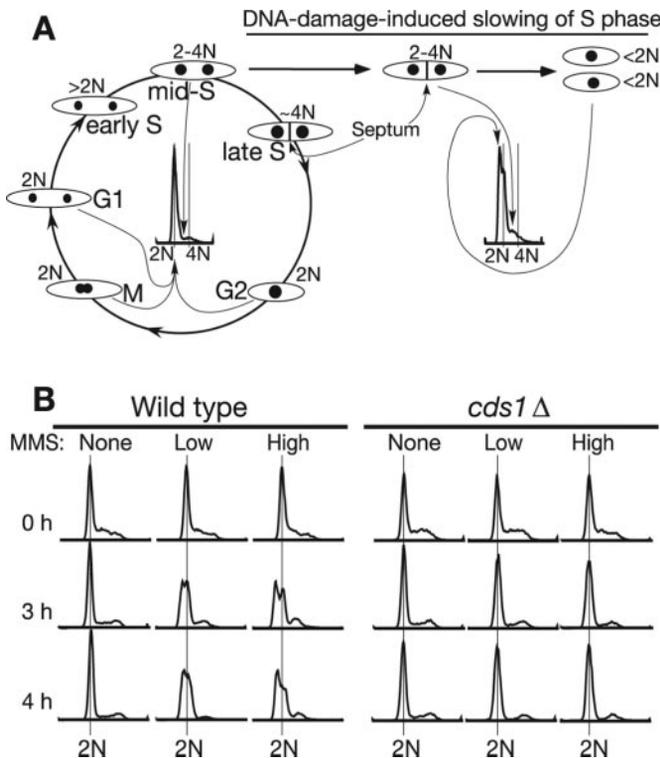


FIG. 1. Demonstration of the intra-S-phase checkpoint in unsynchronized fission yeast cells. A, schematic representation of the *S. pombe* cell cycle. In *S. pombe*, G_2 is the longest cell cycle phase. Cells undergo mitosis, and subsequently binucleate G_1 cells enter S phase. Cytokinesis is temporally separated from mitosis. Mitosis is under checkpoint regulation, but cytokinesis is not. Cytokinesis takes place at a relatively fixed time after mitosis, usually near the end of S phase. Because the timing of cytokinesis is independent of the timing of S phase, when replication is slowed, cytokinesis usually takes place within rather than at the end of S phase. As a consequence of cytokinesis within S phase, those S phase cells (mostly in the later portion of S phase) that have completed cytokinesis have single nuclei with DNA contents between 1N and 2N. The S phase cells (mostly in the early portion of S phase) that have not yet completed cytokinesis retain two nuclei. The total amount of DNA in such cells ranges from 2N to 4N. Note that slowing replication will lead to increased relative abundance of S phase cells, both uninucleate and binucleate. The greater the slowing of S phase, the larger the fraction of S phase cells that will complete cytokinesis and become uninucleate. Thus, a strong intra-S-phase checkpoint response should lead to significant accumulation of uninucleate S-phase cells to the left of the 2N peak, whereas a weaker response should produce less dramatic accumulation of uninucleate S-phase cells (left of the 2N peak) and binucleate S-phase cells (right of the 2N peak). B, demonstration of the MMS-induced intra-S-phase checkpoint in wild type but not in *cds1* Δ cells. Log-phase cells were exposed or not to MMS for the indicated number of hours. Low MMS = 0.0075%; high MMS = 0.015%. Wild type cells developed an obvious shoulder or peak (left of the 2N peak; DNA content 1N-2N) upon MMS treatment, whereas *cds1* Δ cells did not. All samples showed a relatively high proportion of S phase cells at the 0-h time point as a consequence of dilution into fresh medium 1 h previously (see "Experimental Procedures").

cells are in G_2 phase, the G_1 cells (which are binucleate and, thus, have a cellular DNA content of 2N) also contribute to the 2N peak in flow cytometry, and the S phase cells form a shoulder between 2N and 4N, to the right of the 2N peak (Fig. 1, A and B, 0-h time point).

When MMS is added, the majority of checkpoint-competent cells, which were in G_2 before MMS addition, go through mitosis and then enter S phase. When the replication forks in these cells encounter DNA damage, progression through S phase slows down. Because cytokinesis occurs at a fixed time after mitosis regardless of the length of S phase, when S phase is prolonged most cells undergo cytokinesis while they are still in S phase, thus generating a population of uninucleate S-

phase cells. This population forms a shoulder or peak with DNA content less than 2N to the left of the 2N position (Fig. 1, A and B, Wild type, compare low and high MMS with no MMS at the 3- and 4-h time points). Cells deficient in the intra-S-phase checkpoint cannot slow their progression through S phase, and they maintain a normal log-phase-like flow profile even in the presence of MMS. An example is provided in Fig. 1B (*cds1* Δ panel). The advantages of this simple technique are that (i) potential artifacts arising from synchronization are eliminated, and (ii) there are no restrictions (other than ability to produce an interpretable log phase flow profile) on the types of mutant strains that can be investigated. To enhance detection of partial phenotypes, we used two different concentrations of MMS (low (0.0075%) and high (0.015%)) that produced dose-dependent accumulation of wild type cells in S phase (Fig. 1B).

Importance of Cdc2 Phosphorylation for the Intra-S-phase Checkpoint—The Cdc2 protein is the *S. pombe* cyclin-dependent kinase (Cdk) that, together with different cyclins, promotes cell-cycle transitions by phosphorylating important cell cycle regulators. The heterodimer of Cdc2 and the cyclin Cig2 (Cdc2/Cig2) promotes the G_1 /S transition and is normally responsible for initiation of DNA replication (31). On the other hand, Cdc2/Cdc13 promotes passage through mitosis (32). In addition to its essential roles in mitosis and DNA replication, Cdc2 is the downstream effector of the S_{dNTP} -M checkpoint and the G_2 -M checkpoint (the checkpoint that arrests the cell cycle prior to mitosis after DNA damage in G_2) (33, 34).

To investigate the possible role of Cdc2 in the intra-S-phase checkpoint, we used a strain that carries the *cdc2Y15F* mutation. As a consequence of this mutation, tyrosine is replaced by non-phosphorylatable phenylalanine at position 15. The encoded protein, Cdc2Y15F, is a constitutively active cyclin-dependent kinase (35), and the mutant strain exhibits a "wee" (small cell) phenotype as a result of premature mitosis. *S. pombe* cells expressing Cdc2Y15F have previously been demonstrated to be defective in both the S_{dNTP} -M and the G_2 -M checkpoints (33, 34, 36).

We tested the *cdc2Y15F* strain for the intra-S-phase checkpoint using the method described above. Fig. 2C shows the results obtained. In the 2-h MMS-treated samples, this strain showed a slight accumulation of S-phase cells (to the left and right of the 2N peak; see Fig. 1A for interpretation) upon MMS treatment. This small accumulation of S-phase cells mostly disappeared by 4 h. In contrast, robust accumulation of S-phase cells (especially to the left of the 2N peak) was evident in the wild type control cells (Fig. 2A). Thus, in comparison to wild type, *cdc2Y15F* cells have an intra-S-phase checkpoint that is both weak and transient. These results indicate that phosphorylation of Cdc2 at Tyr-15 is a key step in the MMS-induced intra-S-phase checkpoint as well as in the S_{dNTP} -M and G_2 -M checkpoints.

Because Cdc25 is an activator of Cdc2, we hypothesized that a strain overexpressing Cdc25 should have a phenotype similar to that of the *cdc2Y15F* strain. For this purpose we employed a Cdc25-overexpressing strain that had previously been shown to have reduced phosphorylation at Cdc2-Tyr-15 (37). This strain had also been reported to be sensitive to hydroxyurea and deficient in the S_{dNTP} -M checkpoint (38, 39). Consistent with our hypothesis, the Cdc25-overexpressing strain showed only weak, transient accumulation of sub-2N cells 2 h after MMS treatment (Fig. 2B), similar to the phenotype of the *cdc2Y15F* strain (Fig. 2C). Thus, two independent genetic modifications that caused constitutive activation of Cdc2 kinase both resulted in major loss of the intra-S-phase checkpoint.

The tyrosine kinases Wee1 and Mik1 are responsible for phosphorylating Tyr-15 of Cdc2 (26). We predicted that, be-

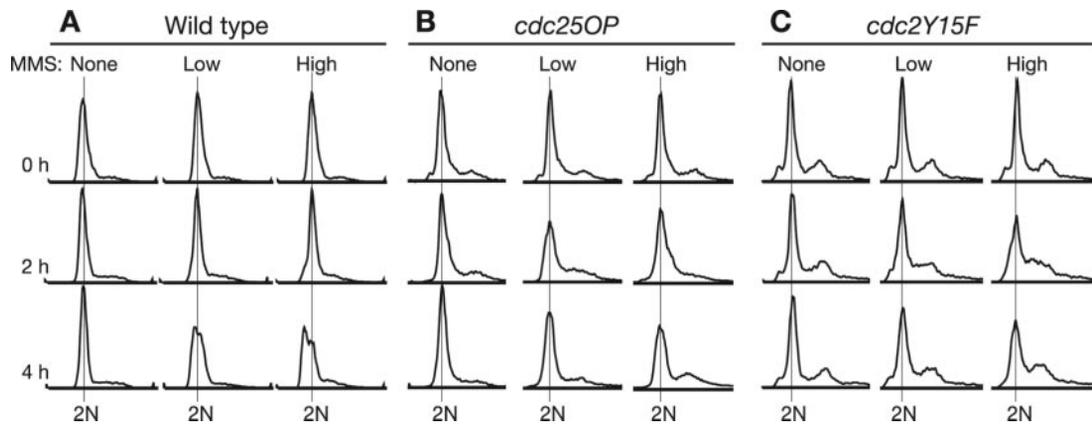


FIG. 2. **Effect of constitutive Cdc2 activation on the intra-S-phase checkpoint.** The experimental procedure was similar to that of Fig. 1B. Cells that hyper-activated Cdc2 kinase either by overproducing Cdc25 phosphatase (B) or expressing non-phosphorylatable *cdc2Y15F* (C) showed a slight accumulation of <2N and >2N cells at 2 h, and this accumulation largely disappeared by 4 h. This differs from the wild type cells (A), where the <2N accumulation was robust and persistent.

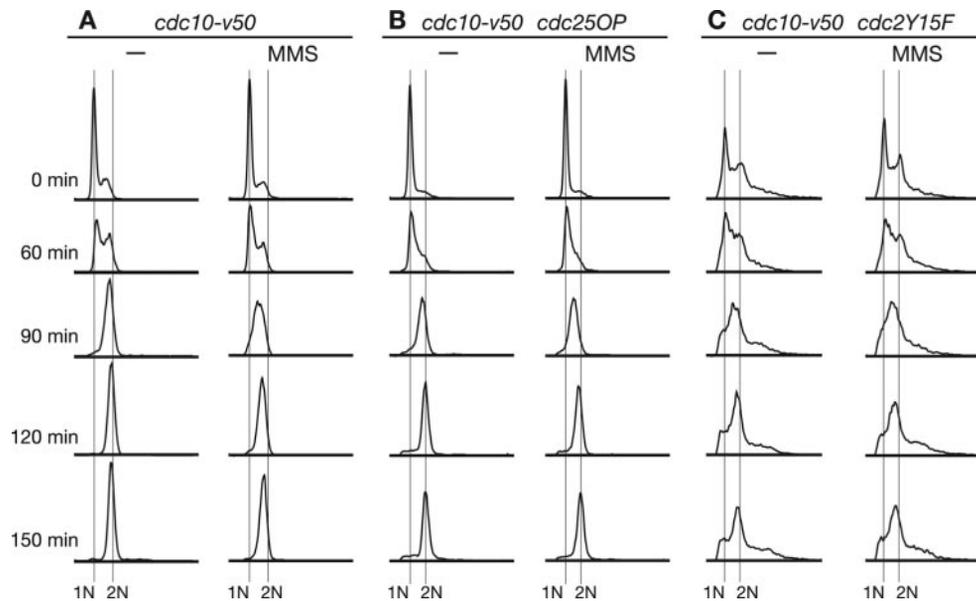


FIG. 3. **Effects of constitutive Cdc2 activation on the intra-S-phase checkpoint in cells synchronized by *cdc10* block and release.** G_1 -arrested cells were released by shifting them to 25 °C. At the time of release 0.015% MMS was added to the panels labeled MMS. A, the *cdc10-v50* cells slowed their progression through S phase upon MMS treatment. B, and C, in contrast, S-phase progression in cells overexpressing Cdc25 (B) or carrying the *cdc2Y15F* allele (C) was not significantly retarded in the presence of MMS.

cause overproduction of Cdc25 (which removes phosphate from Tyr-15) can diminish the intra-S-phase checkpoint (Fig. 2B), then inhibition of Wee1 and Mik1 (which add phosphate onto Tyr-15) should have a similar effect. We found that deletion of *mik1*⁺ alone had no effect on the checkpoint (results not shown). The gene encoding the essential Wee1 protein cannot be deleted, so we studied cells bearing a temperature-sensitive mutant allele, *wee1-50*, at various temperatures. At permissive temperature (25 °C) both *wee1-50* single-mutant cells and *wee1-50 mik1Δ* double-mutant cells were as competent as wild type cells for the intra-S-phase checkpoint (results not shown). Unfortunately, at 30 °C or higher temperatures the single- and double-mutant cells became sick and did not produce interpretable flow cytometry data even in the absence of MMS (results not shown). Thus, our observations show that in the presence of the Wee1 kinase, phosphorylation of Cdc2-Tyr-15 by the Mik1 kinase is not important for the intra-S-phase checkpoint. We suspect that in the absence of Mik1 kinase Wee1 kinase is responsible for inhibitory phosphorylation of Cdc2-Tyr-15 during the intra-S-phase checkpoint response. However, the essential nature of Wee1 prevented us from testing this possibility.

To gain more confidence in our results obtained with the *cdc2Y15F* and the *cdc25OP* strains, we decided to test our conclusions using a synchronization technique based on temperature block and release of *cdc10* mutants. The Cdc10 protein is a transcription factor, and its function is required for the G_1/S transition (40). For this reason, *cdc10* mutant cells arrest in G_1 at non-permissive temperature (35 °C). When the temperature is reduced to 25 °C, the cells enter and pass through S phase synchronously.

We found that when the *cdc10-v50*, *cdc10-v50 cdc25OP*, and *cdc10-v50 cdc2Y15F* strains were incubated at 35 °C for 4 h, most cells accumulated with a 1N DNA content, consistent with arrest in G_1 (Fig. 3, 0 min time point). The *cdc10-v50 cdc2Y15F* strain displayed a broader profile due to its inherent cell cycle defects; nevertheless, a 1N peak was evident. The arrested cultures were shifted to 25 °C and treated or not with 0.015% MMS. Samples for flow cytometry were collected every 30 min. The *cdc10-v50* cells (wild type) slowed their progression through S phase upon MMS treatment (Fig. 3A). In this case the untreated culture completed S phase by 120 min, whereas the MMS-treated cells had not completed S phase by

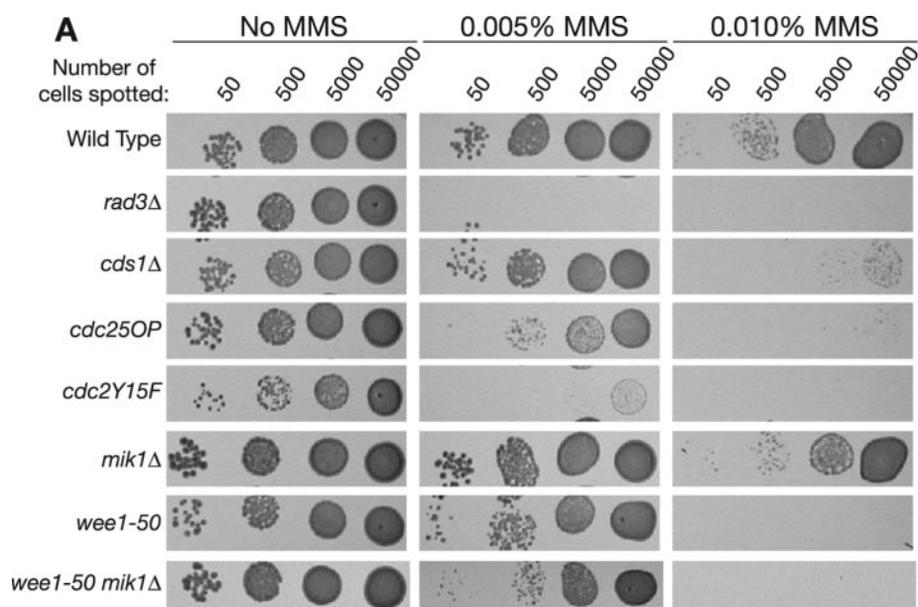
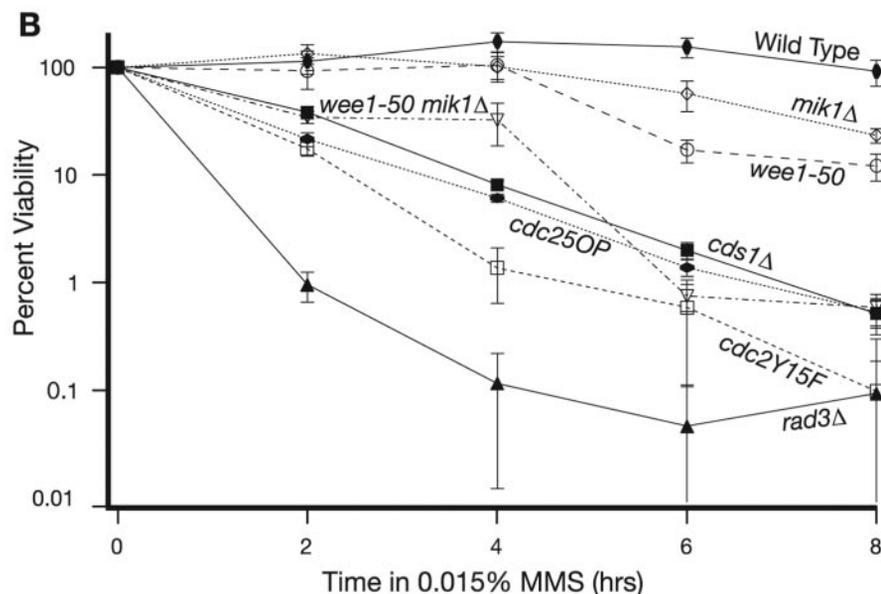


FIG. 4. MMS sensitivities of various mutant strains. *A*, relative abilities of mutant strains to grow in the presence of MMS. 10-Fold serial dilutions of log phase cultures were spotted on YES plates containing the indicated concentrations of MMS. After 4 days at 25 °C, the plates were photographed. *B*, viabilities of different mutants after increasing lengths of exposure to MMS. Log phase cultures were exposed to MMS, and plating efficiency was determined at each time point. For each time point in this single experiment, the logarithm of the percent viability is shown along with a 95% confidence interval (twice the S.D.) based on the number of colonies counted (see “Experimental Procedures”). The results in *A* and *B* show that deletion of *mik1*⁺ had little effect on viability, consistent with this deletion having no detectable effect on the MMS-induced intra-S-phase checkpoint (see “Results”). The *wee1-50*, *cds1Δ*, *cdc25OP*, and *wee1-50 mik1Δ* strains exhibited moderate sensitivity to MMS, whereas the *rad3Δ* and *cdc2Y15F* strains were hypersensitive.



150 min. However, the cells overproducing Cdc25 or expressing Cdc2Y15F went through S phase nearly as rapidly in the presence of MMS as in its absence (Fig. 3, *B* and *C*). Untreated *cdc10-v50 cdc25OP* cells finished S phase by 120 min, whereas the MMS-treated culture finished S phase by 150 min. The untreated *cdc10-v50 cdc2Y15F* cells finished most of S phase by 150 min, and the treated cells were not far behind. These results are consistent with the results in Fig. 2, and they indicate that the intra-S-phase checkpoint in *cdc2Y15F* and *cdc25OP* strains is largely disrupted.

It is important to point out that we did see transient accumulation of small amounts of S-phase cells for both the *cdc2Y15F* and *cdc25OP* strains at the 2-h time point in Fig. 2. In Fig. 3 we observed a slight delay in S-phase progression at the 90-min time point for the *cdc25OP* cells and at 120 min for the *cdc2Y15F* cells. These observations suggest that the intra-S-phase checkpoint deficiency in these strains is not absolute and that the Cdc2-mediated pathway is a major, but may not be the sole contributor to the MMS-induced intra-S-phase checkpoint.

Weak Correlation between Lack of Intra-S-phase Checkpoint and Viability—It is frequently assumed that retardation of S phase upon treatment with a DNA-damaging agent protects cells against loss of viability. To test this assumption, we employed two assays to compare the effects of the mutations discussed above on the viability of fission yeast cells in the presence of MMS; (i) we measured the survival of serial dilutions of wild type and mutant strains on plates containing MMS, and (ii) we measured the plating efficiencies of cells after increasing times of exposure to MMS in liquid culture. If the intra-S-phase checkpoint were the sole determinant of ability to survive MMS damage, we would expect to find a strict correlation between the impact of a mutation on the intra-S-phase checkpoint and its impact on viability. Instead we found (Fig. 4) that there is only a partial correlation between the ability of a cell to slow S phase upon DNA damage by MMS and its ability to survive the damage. All the strains deficient in the intra-S-phase checkpoint were sensitive to MMS but to different extents. The *rad3Δ* and *cdc2Y15F* cells were hypersensitive to MMS, but the *cds1Δ* strain, which is equally deficient in the

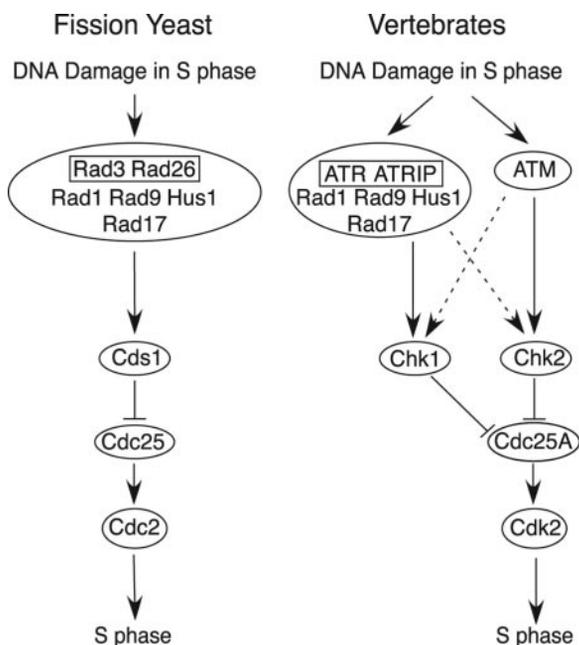


FIG. 5. **Models for the intra-S-phase checkpoint in fission yeast and vertebrates.** The fission yeast model is based on the data presented here and in Marchetti *et al.* (10). The vertebrate model is derived from Abraham (54), Bao *et al.* (55), Falck *et al.* (3), Heffernan *et al.* (5), Kobayashi *et al.* (56), and Weiss *et al.* (57). Both models are simplified to show only the key proteins relevant to intra-S-phase regulation of cyclin-dependent kinases. The vertebrate model shows only the proteins homologous to the ones tested by us in fission yeast.

checkpoint (Fig. 1, (10)) was relatively resistant. The *wee1-50 mik1* Δ double-mutant strain was about as sensitive to MMS as the *cds1* Δ strain in both assays (at permissive temperature) even though the *wee1-50 mik1* Δ strain displayed no checkpoint defect (not shown). The simplest explanation of these observations is that each of the proteins encoded by the genes tested in Fig. 4 contributes to a variable extent to slowing S phase in response to DNA damage by MMS, and each protein probably also contributes to survival of damage by additional mechanisms that are independent of the rate of passage through S phase. Some of these survival mechanisms probably operate outside of S phase. Of the tested proteins, Cds1 is probably the most specific to the intra-S-phase checkpoint because Cds1 is known to function primarily in S phase (9). Even Cds1, however, probably serves additional functions beyond the slowing of S phase, such as promoting the recovery of stalled replication forks (19, 41–43). Thus, the strongest conclusion we can reach is that, if the intra-S-phase checkpoint contributes to survival of MMS damage at all, that contribution cannot be greater in magnitude than the mild MMS sensitivity produced by deletion of the *cds1* gene. The *cds1* deletion completely abrogates the intra-S-phase checkpoint. No other mutation has a stronger effect on the checkpoint than *cds1* Δ , although some mutations, such as *rad3* Δ , have equally strong effects. Consequently, mutations in other genes, such as *rad3* and *cdc2*, which have much stronger effects on the abilities of cells to survive MMS damage, must target additional mechanisms that are independent of the intra-S-phase checkpoint and that are more important than the intra-S-phase checkpoint for damage survival.

DISCUSSION

Parallel between Fission Yeast and Vertebrate Intra-S-phase Checkpoint Pathways—Our results demonstrate an interesting parallel between the vertebrate and fission yeast intra-S-phase checkpoint pathways. In both cases a cyclin-dependent kinase is a downstream effector of S-phase slowing (Fig. 5). The down-

stream effectors of the intra-S-phase checkpoint in budding yeast have not yet been clearly identified; so far there has been no report of a similar role for the budding yeast cyclin-dependent kinase (Cdc28) in slowing S phase upon DNA damage. Regulation of Cdc28 plays only a partial role in the mitotic exit checkpoint (44). Moreover, Mih1p, the Cdc25 homolog in budding yeast, is dispensable for cell viability; its loss causes only subtle defects in the cell cycle (45). Thus, our studies suggest that fission yeast may be a better model than budding yeast for some of the downstream portions of vertebrate S-phase checkpoint responses.

Extensive Identity of Fission Yeast S_{dNTP-M} and the Intra-S-phase Checkpoint Pathways—Our results are consistent with and extend our previously stated (10) hypothesis that, in fission yeast, the initial step in both the hydroxyurea-induced S_{dNTP-M} checkpoint and the MMS-induced intra-S-phase checkpoint is the pausing of replication forks, and most of the downstream steps are also identical. Previously we showed that these two checkpoints appear to utilize identical mechanisms from replication fork pausing to activation of Cds1 kinase (10), but at that time the portions of the intra-S-phase checkpoint downstream of Cds1 were unknown. Our current results show that a major portion of the MMS-induced intra-S-phase checkpoint downstream of Cds1 uses the same mechanism (inhibition of Cdc2 kinase) as the hydroxyurea-induced S_{dNTP-M} checkpoint. Further studies are needed to determine exactly how inhibition of Cdc2 slows S phase and also to determine the extents to which other proteins that are important for the intra-S-phase checkpoint contribute to inhibition of Cdc2 or participate in parallel pathways. Recently, mutants in the fission yeast MRN complex have been shown to be defective in the intra-S-phase checkpoint (18). These mutants so far have not been tested for possible defects in the S_{dNTP-M} checkpoint. In another study Swi1 was shown to be required for activation of Cds1 upon hydroxyurea treatment (19). Thus, Swi1 is important for the S_{dNTP-M} checkpoint. It remains to be seen if Swi1 is similarly required for the intra-S-phase checkpoint. Additional evidence for the identity of the S_{dNTP-M} checkpoint and the intra-S-phase checkpoint is provided by *hsk1-89* and *mrc1* Δ cells. Both of these strains are defective in the S_{dNTP-M} checkpoint (46–48) as well as the intra-S-phase checkpoint.²

Lack of Correlation between Damage Survival and Competence in the Intra-S-phase Checkpoint—Our observations indicate that, although the intra-S-phase checkpoint may contribute to cell survival after MMS treatment, other processes that are independent of the intra-S-phase checkpoint are considerably more important for survival. Some of these other processes are dependent on functions of Rad3 and Cdc2 that are not relevant to the intra-S-phase checkpoint. Our results in fission yeast are consistent with the finding in budding yeast that slowing of S phase by inhibition of initiation at late origins contributes significantly less to MMS survival than does stabilization of stalled replication forks (49). However, it has not yet been established in fission yeast that slowing of S phase is accomplished by inhibiting initiation at late origins nor has it been demonstrated that the ability of fission yeast cells to survive MMS treatment depends primarily on replication fork stabilization. In other words, the mechanisms that render the intra-S-phase checkpoint a relatively minor contributor to survival of both budding and fission yeast may prove to differ between the two organisms.

The relative lack of importance of the intra-S-phase checkpoint for survival of DNA damage does not mean that the checkpoint is not important for the preservation of genome

² S. Kumar and J. Huberman, unpublished results.

stability. Indeed, reduction of genome stability (in contrast to reduction of viability) may account for the striking correlation between loss of the intra-S-phase checkpoint and cancer in humans (2).

Does Cdc25 Play a Role in the Regulation of Unperturbed S Phase?—Cdc25 phosphatase activity increases dramatically at the end of G₂ and is essential for entry into mitosis (50–52). Cells with temperature-sensitive mutations in the *cdc25* gene arrest in late G₂ (just before mitosis) at the non-permissive temperature (10). There is no detectable accumulation of cells in S phase under such conditions (10). For these reasons, the Cdc25 phosphatase has previously been considered to be important for regulation of mitosis but not of S phase. Our results now show, however, that inhibition of Cdc25 during S phase (as part of the intra-S-phase checkpoint) can significantly slow progression through S phase. This result implies that the normal rate of progression through S phase may be regulated in part by the level of Cdc25 activity even in the absence of DNA damage. Consistent with this possibility, the rate of DNA replication in *Xenopus* egg extract is diminished by a specific inhibitor of Cdc25A (53).

This possibility raises the question of why there is no detectable accumulation of S phase cells in *cdc25* temperature-sensitive strains at non-permissive temperatures. A plausible explanation is that, when the temperature is shifted from permissive to non-permissive, mitosis would be inhibited rapidly (since the level of Cdc25 phosphatase activity would rapidly drop below the high threshold required for mitosis), whereas S phase would be inhibited relatively slowly (since lower Cdc2 kinase levels, hence, lower Cdc25 phosphatase levels are sufficient to support S phase (25)). These kinetics would permit all of the G₁ and S phase cells in an unsynchronized population of *cdc25* temperature-sensitive mutant cells to complete G₁ and S phase after shift to the non-permissive temperature without accumulating. These cells, which were originally in G₁ and S, would then become arrested in late G₂ just like the other cells in the population.

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