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Rfc5, a Replication Factor C Component, Is Required for Regulation of Rad53 Protein Kinase in the Yeast Checkpoint Pathway

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The *RFC5* gene encodes a small subunit of replication factor C (RFC) complex in *Saccharomyces cerevisiae*. We have previously shown that a temperature-sensitive (ts) *rfc5-1* mutation is impaired in the S-phase checkpoint. In this report, we show that the *rfc5-1* mutation is sensitive to DNA-damaging agents. *RFC5* is necessary for slowing the S-phase progression in response to DNA damage. The phosphorylation of the essential central transducer, Rad53 protein kinase, is reduced in response to DNA damage in *rfc5-1* mutants during the S phase. Furthermore, the inducibility of *RNR3* transcription in response to DNA damage is dependent on *RFC5*. It has been shown that phosphorylation of Rad53 is controlled by Mec1 and Tel1, members of the subfamily of ataxia-telangiectasia mutated (ATM) kinases. We also demonstrate that overexpression of *TEL1* suppresses the ts growth defect and DNA damage sensitivity of *rfc5-1* mutants and restores phosphorylation of Rad53 and *RNR3* induction in response to DNA damage in *rfc5-1*. Our results, together with the observation that overexpression of *RAD53* suppresses the defects of the *rfc5-1* mutation, suggest that Rfc5 is part of a mechanism transducing the DNA damage signal to the activation of the central transducer Rad53.

In eukaryotic cells, successful mitotic division requires the events of the cell cycle to be ordered into dependent pathways in which the initiation of late cycle events is dependent on the completion of early events. The mechanisms which ensure that cell division does not occur before completion of such prerequisite steps have been termed checkpoint controls (8). Checkpoint controls ensure that cells remain in S phase before completion of DNA replication. DNA damage also activates checkpoint controls to provide enough time to complete DNA repair. Defects of these DNA-related checkpoints result in increased genomic instability and mutagenesis (5, 21).

In the budding yeast *Saccharomyces cerevisiae*, checkpoint pathways induce cell cycle arrest in G_1 or G_2/M and retard S-phase progression in response to DNA damage. Other checkpoints prevent cells with incompletely replicated DNA from exiting the S phase (5, 21). A number of genes that are involved in the DNA damage checkpoint and/or the replication checkpoint have been identified (5, 21). These include *RAD9*, *RAD17*, *RAD24*, *POL2*, *MEC1*/*ESR1*, *RAD53*/*SPK1*/*MEC2*/*SAD1*, and *MEC3* (1, 10, 17, 28, 33–35). Among these genes, *RAD9*, *RAD17*, *RAD24*, and *MEC3* are involved not only in the G_2/M-phase but also in the G_1- and S-phase DNA damage checkpoints (12, 20, 25–27, 33–35). *POL2*, which encodes a large subunit of DNA polymerase ε (pol ε), is proposed to sense DNA damage and replication block in S phase (16, 17). *MEC1* and *RAD53* are necessary for checkpoints operating in response to both DNA damage and incomplete DNA replication (1, 35). *RAD53* encodes a dual-specificity protein kinase (28), and Mec1 belongs to the phosphatidylinositol kinase family that includes *S. cerevisiae* Tel1 and human ATM proteins (10, 15, 24). *MEC1* and *TEL1* share some overlapping functions in checkpoint controls, although *tel1*Δ mutants themselves are not defective in checkpoint functions (15, 23).

Checkpoint pathways are predicted to have at least three components: a monitoring system to detect the change in DNA structure, a signal pathway to transmit the information from this monitoring system to the cell cycle machinery, and a target leading to cell cycle delay as a consequence of DNA damage or replication block. It has been demonstrated that Rad53 is phosphorylated in response to DNA damage and replication block, thus correlating the activation of checkpoint pathways to a biochemical modification of a checkpoint protein. This modification is dependent on *MEC1* because the phosphorylation of Rad53 does not occur in *mec1* mutants. Furthermore, genetic analysis has revealed that overexpression of *RAD53* can suppress the sensitivity to the DNA replication inhibitor hydroxyurea (HU) and the lethality of *mec1* mutations (23). This epistatic relationship indicates that Rad53 functions downstream of Mec1 and may be directly phosphorylated and activated by Mec1.

The *RFC5* gene encodes a small subunit of the RFC complex in *S. cerevisiae* (4, 30). A temperature-sensitive (ts) mutant of *RFC5* whose lethality can be suppressed by overexpression of the Rad53 kinase has been identified (30). At the restrictive temperature, *rfc5-1* mutant cells enter mitosis with unevenly separated or fragmented chromosomes, resulting in a loss of viability. The *rfc5-1* phenotype appears to be the combined result of a DNA replication defect and a failure of the checkpoint control that prevents the onset of mitosis before DNA replication has been completed. In this study, we show that *rfc5* mutants are sensitive to DNA-damaging agents. Furthermore, *RFC5* is necessary for slowing of DNA synthesis when DNA is damaged during replication. To investigate the signaling of DNA damage, we examined the ability of *rfc5-1* mutants to phosphorylate Rad53 and activate transcription of the damage-inducible *RNR3* in response to DNA damage. The results presented here raise the possibility that Rfc5 is part of a mechanism that senses and transduces the DNA damage signal, leading to the activation of Rad53 protein kinase.

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TABLE 1. Strains used in this study

Strain[a]	Genotype
KSC766	*MAT***a** *rfc5-1 ade2 his2 trp1 ura3 leu2 lys2*
KSC800	*MAT***a** *rfc5-1::RFC5::LEU2 ade2 his2 trp1 ura3 leu2 lys2*
KSC953	*MAT***a** *rfc5-1 ade2 his3 trp1 ura3 leu2*
KSC1004	*MAT***a** *rfc5-1 tel1Δ::HIS3 ade2 his3 trp1 ura3 leu2*

[a] KSC766 is isogenic to KSC800 and congenic to KSC953 and KSC1004.

MATERIALS AND METHODS

Strains, media, and general methods. The yeast strains used in this study are described in Table 1. DNA was manipulated by standard procedures (22). Standard genetic techniques were used for manipulating yeast strains (9). The media used to maintain selection for *TRP1* and *URA3* plasmids are synthetic complete media containing 0.5% Casamino Acids and the appropriate supplements.

Plasmid constructions. The 3.5-kb *Eco*RI fragment from YEpRAD53 (30) was cloned into *Eco*RI-digested YCplac33 (6), creating YCp33-RAD53. The DNA sequences encoding the epitope recognized by the antihemagglutinin (anti-HA) monoclonal antibody 12CA5 were attached in frame to the C-terminal end of *RAD53* by PCR. The C-terminal *RAD53* open reading frame was amplified by PCR with the 5′ primer CTCTCTAGAATACCCGCAGACGCCCCT and the 3′ primer CGGATCCCCGAAAATTGCAAATTCTC. The *Xba*I-*Bam*HI-digested PCR fragment and the *Bam*HI-*Xho*I fragment containing DNA sequences of two HA epitope tags (YPYDVPDYA) were subcloned into pBluescript (−) to create pKS159. The *Sal*I fragment of YCp33-RAD53 was replaced by a 1.2-kb *Sal*I fragment from pKS159, creating YCp-RAD53-HA. The tagged construct (*RAD53-HA*), when expressed from its own promoter and carried on the YCp plasmid, fully complemented a null mutation (*rad53Δ::LEU2*) with regard to growth rate and sensitivity to HU and DNA-damaging agents, such as methyl methanesulfonate (MMS) and UV irradiation. YEpMEC1 was generated by subcloning *Sac*II-*Nhe*I and *Nhe*I-*Hpa*I fragments from the *MEC1* gene (obtained from T. Weinert) into *Sac*II-*Sma*I-treated pRS426 (3). YEpMEC1 rescued the *mec1* mutation. pDM198 (YEpTEL1) is a derivative of pRS426 carrying the *TEL1* gene (15). YEpT-TEL1 was generated by subcloning the *Not*I-*Sal*I fragment from pDM197 (15) into pYO324, a *TRP1*-marked YEp vector (18). YEpPOL30 and YEpT-POL30 were constructed by subcloning the *Bam*HI-*Xba*I fragment of the *POL30* gene into YEplac195 (6) and YEplac112 (6), respectively. YCpRFC5 is a YCplac33 carrying *RFC5* (30).

Western blotting. Yeast cells were grown in synthetic complete medium selectable for *URA3* and/or *TRP1* plasmids. The cells were then diluted in yeast extract-peptone-dextrose (YEPD) and allowed to grow for 3 h before being temperature shifted and treated with HU or MMS. For arrest with nocodazole, the cells were incubated with 20 μg of nocodazole per ml at 25°C for 150 min, shifted to 37°C for 1 h, and then treated with MMS at 37°C for 2 h. The cells (optical density at 600 nm = 10) were pelleted, washed, and resuspended in sodium dodecyl sulfate (SDS) sample buffer U (40 mM Tris-HCl [pH 7.0], 0.1 mM EDTA, 4% SDS, 8 M urea). An equal volume of glass beads was added, and the cells were lysed by vortexing. Extracts were clarified by 15 min of centrifugation. After the addition of 2-mercaptoethanol to 1%, the samples were boiled for 5 min and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (9% polyacrylamide). The proteins were then transferred to a nylon membrane and subjected to Western blot analysis with the anti-HA monoclonal antibody 12CA5. HA-tagged Rad53 proteins were detected by an enhanced chemiluminescence kit (Amersham).

Protein kinase assay. Yeast cells were grown in synthetic complete medium selectable for *URA3* plasmids, diluted in YEPD, and allowed to grow at 25°C for 3 h. The culture was synchronized in the G_1 phase by addition of 6 μg of α-factor per ml. After 2 h at 25°C, α-factor (6 μg/ml) was added and the culture was split in half and shifted to 37°C for 1 h. One half was collected for the kinase assay after incubation at 37°C, and the other half was treated with HU at 10 mg/ml during the last 30 min of incubation with α-factor and then washed to remove α-factor and released into YEPD containing 10 mg of HU per ml at 37°C. After a 2-h incubation, HU-treated cells were collected for the kinase assay. Cells (optical density at 600 nm = 20) were pelleted, washed, and resuspended in 150 μl of lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM each EDTA, sodium orthovanadate, and dithiothreitol, 0.1% Triton X-100, 40 mM β-glycerophosphate, 15 mM p-NO_2-phenylphosphate, 1 μg each of leupeptin and pepstatin per ml, 0.5% aprotinin, 100 μg of APMSF per ml). An equal volume of glass beads was added, and the cells were lysed by vortexing. Extracts were clarified by 15 min of centrifugation at 4°C. The supernatant was diluted with lysis buffer and incubated at 4°C for 2 h with 30 μl of protein A-Sepharose beads bound with mouse monoclonal 12CA5 anti-HA antibody. The protein concentration was determined by the Bio-Rad protein assay. Immunoprecipitates were washed four times with lysis buffer and twice with kinase buffer (20 mM HEPES-Na [pH 7.5], 10 mM $MgCl_2$, 4 mM $MnCl_2$) and separated into equal portions. Half of each was boiled immediately in 1× sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 4% 2-mercaptoethanol, 10% glycerol) for Western blotting. The other half was used for the kinase assay. The kinase reaction was initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham). Reactions were terminated by the addition of 5× sample buffer and boiling for 5 min. The eluted proteins were separated by SDS-PAGE, and the gels were dried and autoradiographed.

UV radiation and drug sensitivities. The UV radiation sensitivity assay was performed as described previously (29). Cells grown at 37°C were plated on YEPD and then irradiated with UV at 254 nm. After 2 to 3 days of incubation at 37°C, the colony number was counted. MMS sensitivity was determined as described previously (29). The cells were incubated with 0 to 0.45% MMS at 37°C for 10 min. The incubation was terminated by the addition of sodium thiosulfate to a final concentration of 5%. After incubation at 37°C for 2 to 3 days, the number of colonies was counted. For cell cycle arrest experiments, asynchronous cultures were grown to the logarithmic phase and nocodazole (15 μg/ml) was added to the culture at 37°C. After a 2-h incubation, the cells were incubated with 0 to 0.15% MMS at 37°C for 10 min and the incubation was terminated by addition of sodium thiosulfate to a final concentration of 5%. MMS-treated cells were released into medium containing nocodazole to maintain the arrest for a further 2 h. The HU sensitivity assay was performed as described previously (30).

DNA flow cytometry. DNA flow cytometry was performed as described (29) with the modification that the cells were harvested and fixed in 70% ethanol for 12 to 24 h at −20°C. After resuspension in 0.3 ml of 50 mM Tris-HCl (pH 7.5), the cells were sonicated briefly and RNase A was added to a final concentration to 1 mg/ml. Following a 2-h incubation at 50°C, the cells were resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 μg of propidium iodine per ml and 0.5 mg of proteinase K per ml and incubated in the dark for 12 to 48 h at 4°C. Samples were analyzed with a Becton-Dickinson FACScan.

MMS synchrony experiment. For analysis of the S-phase checkpoint, yeast cells were grown in synthetic complete medium selectable for *URA3* plasmids, diluted in YEPD, and allowed to grow at 25°C for 3 h. Samples were removed for DNA flow cytometry analysis, and the remainder of the culture was synchronized in the G_1 phase by the addition of 6 μg of α-factor per ml. After 2 h at 25°C, α-factor (6 μg/ml) was added and the culture was shifted to 37°C for 1 h. The cells were then washed to remove α-factor and released into YEPD with or without 0.05% MMS at 37°C. At the indicated times after release from α-factor, samples were removed for DNA flow cytometry analysis, viability assessment, and Western blot analysis. We confirmed that the peak shifting we observed in flow cytometry is a reflection of chromosomal DNA synthesis. The shift of the flow cytometry histogram from G_1 to G_2/M positions in cells treated with MMS is inhibited by α-factor, which induces G_1 arrest but allows the cells to continue growing, and by HU, which inhibits DNA synthesis. To assess the viability, samples were resuspended in 2% sodium thiosulfate and incubated at 25°C.

Northern blot analysis. Northern blot analysis was performed as described previously (29). The DNA probes were *RNR3*, the 2.1-kb *Mlu*I-*Eco*RI fragment of the *RNR3* gene from pSE734 (1), and *ACT1*, the 1-kb *Xho*I-*Hin*dIII fragment derived from pYS91 containing *ACT1* cDNA (29). The radioactivity of the bands was quantified with a Fuji BAS1000 imaging analyzer.

β-Galactosidase assay. β-Galactosidase assays with the reporter plasmid pZZ13 were performed as described previously (36).

Immunofluorescence microscopic analysis. Yeast cells were grown in synthetic complete medium selectable for *URA3* plasmids, diluted in YEPD, and allowed to grow at 25°C for 3 h. The culture was synchronized in the G_1 phase by the addition of 6 μg of α-factor per ml. After 2 h at 25°C, α-factor (6 μg/ml) was added and the culture was shifted to 37°C for 1 h. HU was added to the culture at 10 mg/ml during the last 30 min of incubation with α-factor. The cells were then washed to remove α-factor and released into YEPD containing 10 mg of HU per ml at 37°C. Aliquots of cells were removed and processed for indirect immunofluorescence microscopy as described previously (29).

RESULTS

Sensitivity of *rfc5-1* mutants to DNA damage. The ts *rfc5-1* mutation is defective in DNA replication at the restrictive temperature. We have shown that overexpression of *POL30*, the gene encoding the proliferating-cell nuclear antigen (PCNA), suppressed the ts growth defect of *rfc5-1* mutants but still allowed the *rfc5-1* cells to enter mitosis following treatment with HU at 37°C, resulting in a rapid loss of viability (30). Thus, PCNA overexpression appears to suppress the DNA replication defect but not the S-phase checkpoint defect in *rfc5-1* cells. The cellular response to DNA damage has many features in common with the response to DNA replication interference. The DNA damage and S-phase checkpoint pathways share overlapping regulatory components such as *MEC1* and *RAD53* (1, 35). In this regard, we tested the *rfc5-1* mutation for its responses to DNA-damaging agents, such as MMS and UV irradiation. *rfc5-1* mutants were not sensitive to UV irradiation or MMS treatment at the permissive temperature

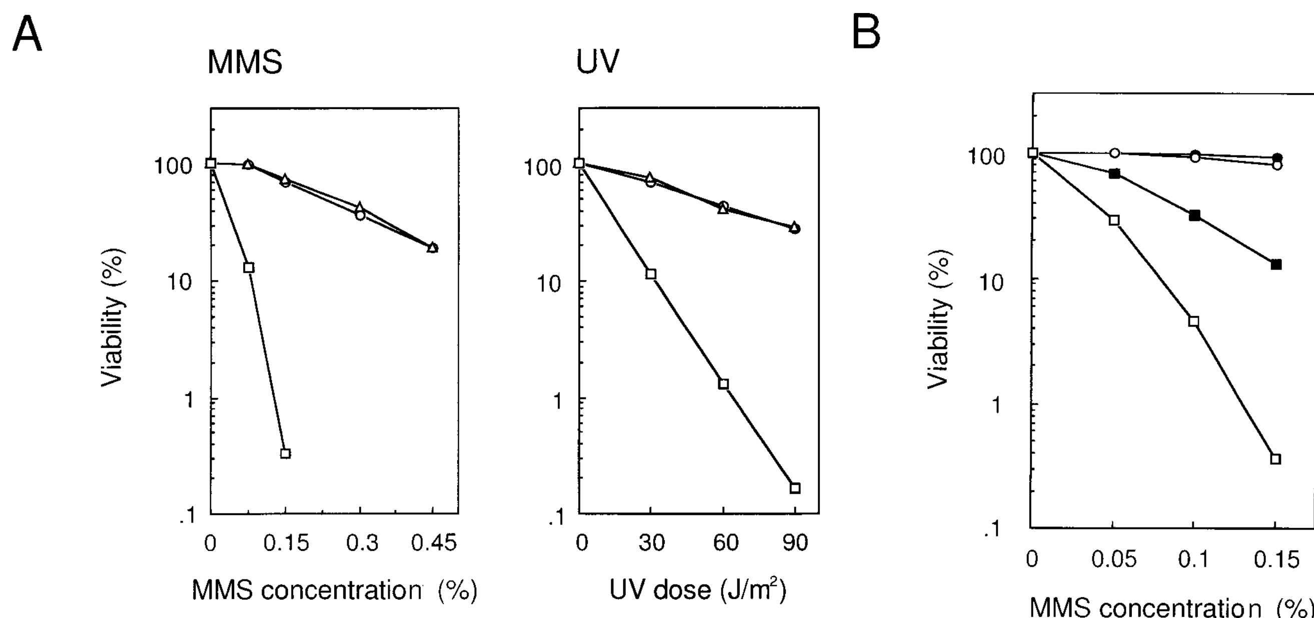


FIG. 1. Sensitivity of *rfc5* mutants to MMS or UV. (A) The *rfc5-1* mutant (KSC766) cells were transformed with YEp195-POL30 (□), and *RFC5*$^+$ (KSC800) cells were transformed with YEp195-POL30 (△) or YEplac195 (○). For MMS treatment, log-phase cells grown in medium selectable for *URA3* plasmids at 37°C were incubated with the indicated concentrations of MMS for 10 min. For UV treatment, log-phase cells grown at 37°C were irradiated at the indicated doses with UV light. The viability of cells was estimated as described in Materials and Methods. (B) The *rfc5-1* mutant (KSC766) cells were transformed with YEp195-POL30 (□, ■) or YCpRFC5 (○, ●). Cells grown in asynchronous culture at 37°C were treated with the indicated concentrations of MMS for 10 min. For nocodazole block, cells grown in asynchronous culture were incubated with 15 μg of nocodazole per ml at 37°C for 2 h. Cells arrested with nocodazole at 37°C were incubated with the indicated concentrations of MMS for 10 min. MMS-treated cells were released into medium containing nocodazole to maintain the arrest for a further 2 h. Open and solid symbols indicate MMS sensitivities of cells in asynchronous culture and cells arrested with nocodazole, respectively. The viability of cells was estimated as described in Materials and Methods.

(25°C) (data not shown). At the restrictive temperature (37°C), *rfc5-1* mutant cells overexpressing PCNA were significantly sensitive to killing by MMS and UV irradiation compared with the wild-type cells (Fig. 1A). Moreover, overexpression of PCNA did not render wild-type cells sensitive to MMS or UV, indicating that DNA damage sensitivity is associated with the *rfc5-1* mutation. These observations suggest that the *RFC5* gene is involved in DNA repair and/or the DNA damage cell cycle checkpoints.

If DNA damage sensitivity of *rfc5-1* mutants is derived from checkpoint defects, it should be suppressed by prior treatment with nocodazole, which arrests cells in G_2/M and prevents inappropriate cell cycle progression through the damage stage. To test this possibility, *rfc5-1* mutant cells overexpressing PCNA were arrested at G_2/M with nocodazole and then exposed to MMS (Fig. 1B). The number and morphology of the cells were monitored throughout the experiment, confirming that more than 90% of the cells arrested as large budded cells. DNA flow cytometry analysis showed that *rfc5-1* mutant cells overexpressing PCNA were not delayed at G_2/M in the absence of nocodazole at 37°C (data not shown). As shown in Fig. 1B, the viability loss of *rfc5-1* mutant cells in the presence of MMS was partially prevented by holding the cells in G_2/M. This result supports the possibility that the *rfc5-1* mutation is defective in the DNA damage checkpoint. Nocodazole treatment did not restore MMS resistance to levels found in the wild type, which suggests that the *rfc5-1* mutation is also defective in DNA repair.

Effect of the *rfc5-1* mutation on Rad53 phosphorylation in response to DNA damage. Rad53 is an essential protein kinase that plays a pivotal role in the DNA damage checkpoint pathway. Exposure of cells to MMS leads to the phosphorylation of Rad53, resulting in accumulation of a lower-mobility form of Rad53 (23, 31). Overexpression of *RAD53* can suppress the ts growth defect and the sensitivity to MMS in *rfc5-1* mutants (30) (see Fig. 5). This suggests that *RFC5* acts upstream of *RAD53* and regulates its activity in response to DNA damage. We were therefore interested in determining whether the activation of the *RAD53* pathway is dependent on *RFC5*. To test this hypothesis, the DNA damage-regulated in vivo phosphorylation state of Rad53 in wild-type and *rfc5-1* mutant cells expressing the Rad53-HA protein was examined (Fig. 2). When wild-type cells expressing Rad53-HA were treated with MMS at 37°C, Rad53-HA became highly phosphorylated, as indicated by the appearance of isoforms with lower electrophoretic mobility. In contrast, after *rfc5-1* mutants were transferred from 25 to 37°C, the phosphorylation of Rad53-HA in response to MMS treatment was greatly reduced. These observations suggest that the *rfc5-1* mutation is defective in signal transduction for phosphorylation of Rad53 in response to DNA damage. The failure of *rfc5-1* mutants to induce phosphorylation of Rad53 in response to MMS is not a general characteristic of mutants

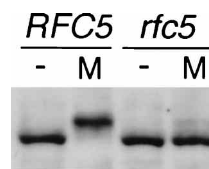


FIG. 2. Modification of Rad53 in response to MMS in *rfc5* mutants. *RFC5*$^+$ (KSC800) and *rfc5-1* mutant (KSC766) cells containing YCp-RAD53-HA were grown at 25°C and shifted to 37°C for 1 h. The cells were then incubated in YEPD (lanes −) or YEPD containing 0.1% MMS (lanes M) at 37°C for 2 h. The cells were subjected to Western blot analysis as described in Materials and Methods.

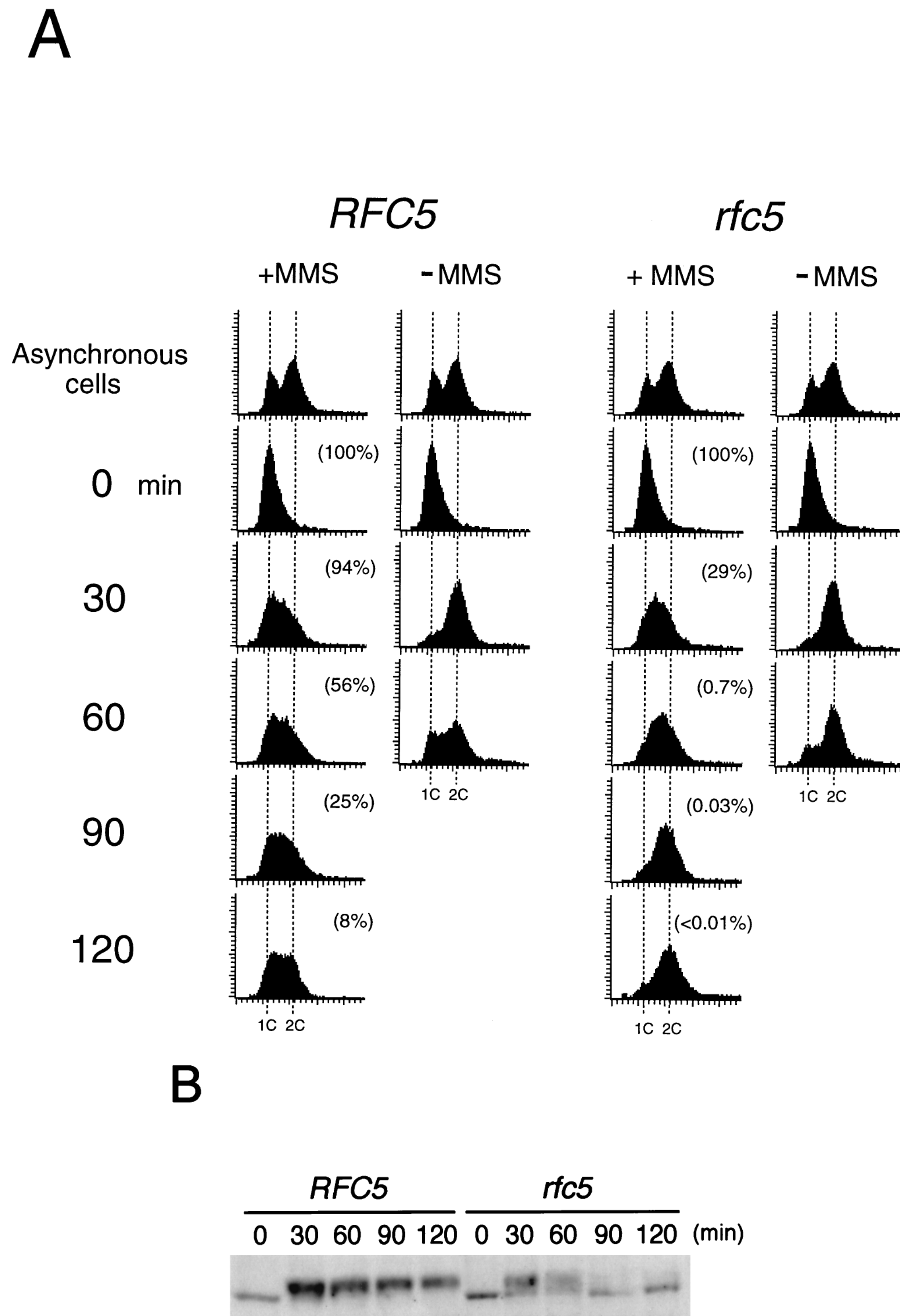


FIG. 3. Kinetics of MMS-induced modification of Rad53 in G_1-synchronized *rfc5* mutants. (A) *RFC5*$^+$ (KSC800) and *rfc5-1* (KSC766) mutant cells carrying YCp-RAD53-HA were synchronized in G_1 and released in either the presence or the absence of 0.05% MMS as described in Materials and Methods. Aliquots of cells were collected at the indicated times after release from α-factor treatment and examined for DNA content by flow cytometry. Dotted lines indicate the DNA content of 1C and 2C cells. The top panels represent asynchronous cells untreated with MMS at 25°C and are included as a reference. The viability of cells at the indicated times after release into MMS is shown in parentheses. (B) Samples at the indicated times after release from α-factor into MMS were subjected to Western blot analysis as described in Materials and Methods.

defective in DNA replication components. For example, *cdc2* (pol δ), *cdc8* (thymidylate kinase), and *cdc17* (pol α) mutants can induce Rad53 phosphorylation even in the absence of MMS treatment when shifted to the nonpermissive temperature (31).

Effect of cell cycle position on Rad53 phosphorylation in response to DNA damage in *rfc5-1* mutants. It has recently been shown that the lowering of the rate of progression through S phase as a result of DNA damage during DNA

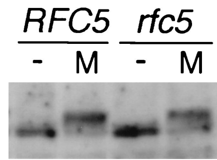


FIG. 4. Modification of Rad53 in *rfc5* mutants at G_2/M. *RFC5*$^+$ (KSC800) and *rfc5-1* mutant (KSC766) cells carrying YCp-RAD53-HA were grown and arrested with 20 μg of nocodazole per ml at 25°C for 150 min. The cells were shifted to 37°C for 1 h and then incubated with YEPD (lanes −) or YEPD containing 0.1% MMS (lanes M) at 37°C for 2 h. Microscopic examination confirmed that during the experiments 90% of cells maintained their large budded phenotype (G_2/M arrest) for nocodazole treatment. The cells were subjected to Western blot analysis as described in Materials and Methods.

replication is controlled by the *RAD53* gene (19). To test the possible involvement of *RFC5* in this control, we analyzed Rad53 phosphorylation and progression through S phase with cells synchronized in the G_1 phase and then released into the cell cycle in either the presence or the absence of MMS (Fig. 3). As observed by Paulovich and Hartwell (19), wild-type cells treated with MMS replicated their DNA more slowly than did untreated control cells. Cells completed replication within 30 min in the absence of MMS, whereas cells replicating in the presence of MMS had still not completed replication 150 min after release from α-factor arrest (Fig. 3A and data not shown). As shown in Fig. 3B, Rad53 was phosphorylated in wild-type cells in response to MMS. *rfc5-1* mutants showed some slowing of S-phase progression in the presence of MMS. However, the degree to which the S-phase progression was slowed in *rfc5-1* mutants was much lower than the degree to which it was slowed in the wild type. For example, 120 min after release into MMS, *rfc5-1* had completed the S phase whereas the wild type still showed significant accumulation in the S phase (Fig. 3A). Hence, *rfc5-1* mutants showed attenuated S-phase regulation in response to MMS. Correspondingly, phosphorylation of Rad53-HA and cell viability in *rfc5-1* mutants were reduced in response to MMS. Modification of Rad53-HA was observed 30 to 60 min after α-factor release into MMS and then decreased 120 min after the release (Fig. 3B). The partial defects in the S-phase regulation in *rfc5-1* mutants could be due to some residual checkpoint activity of the mutation allele. Alternatively, Rfc5 itself would be necessary for only a subset of functions that are necessary for the wild-type level of the S-phase regulation, and checkpoint determinants other than Rfc5 might still be operative.

RAD53 is necessary for DNA damage-induced cell cycle regulation at the G_2/M-phase transition (1, 35). We therefore tested whether *RFC5* is also involved in the G_2/M DNA damage checkpoint. Wild-type and *rfc5-1* mutant cells were prearrested in M phase with nocodazole at 25°C and then treated with MMS at 37°C (Fig. 4). In nocodazole-arrested wild-type and *rfc5-1* cells, Rad53-HA was phosphorylated in response to MMS treatment at 37°C. This suggests that *rfc5* mutants still sense DNA damage with respect to the G_2/M DNA damage checkpoint. The G_2/M DNA damage checkpoint was further examined by arresting cells in M phase with nocodazole at 25°C, treating them with MMS at 37°C, and releasing them from nocodazole at 37°C. Then the rate of cell cycle progression was examined by monitoring nuclear division. Wild-type and *rfc5-1* mutants cells exhibited a similar delay of cell cycle progression in response to MMS treatment relative to the untreated cells (data not shown). These results show that the G_2/M DNA damage checkpoint is intact in the *rfc5-1* mutant cells.

Effect of *RAD53*, *MEC1*, and *TEL1* on the *rfc5-1* mutation. We have shown previously that overexpression of *RAD53* could suppress the ts growth defect of *rfc5-1* mutants (Fig. 5A) (30). Furthermore, *RAD53* overexpression also partially suppressed the DNA damage sensitivity of *rfc5-1* (Fig. 5B). Together with the finding that Rad53 modification by MMS damage is dependent upon *RFC5* (Fig. 2), these results suggest that *RFC5* functions upstream of *RAD53* in the same pathway. The epistatic relationship indicates that *MEC1* and *TEL1* function upstream of *RAD53* in the checkpoint pathways (23). To investigate the relationship between *RFC5* and *MEC1* or *TEL1*, we examined the effects of overexpression of *MEC1* or *TEL1* on the phenotypes of *rfc5-1* mutants. Overexpression of *TEL1* suppressed the ts growth defect of *rfc5-1* mutants and partially restored the ability of *rfc5-1* cells to survive exposure to MMS and UV, whereas *MEC1* overproduction failed to suppress the *rfc5-1* phenotypes (Fig. 5A and B). *RAD53* overexpression was unable to suppress the ts growth defect of *rfc5-1 tel1*Δ double mutants, suggesting that the suppression of *rfc5-1* by *RAD53* overexpression is dependent on the presence of *TEL1* (Fig. 5C).

To establish the genetic interaction between *RFC5* and *TEL1*, we examined the phosphorylation of Rad53 induced by the DNA damage checkpoint in *rfc5-1* mutants overexpressing *TEL1*. MMS-induced phosphorylation of Rad53-HA was not observed in *rfc5-1* mutants with a control vector but could be restored by overexpression of the *TEL1* gene (Fig. 6). These data suggest that *TEL1* overproduction may suppress the *rfc5* checkpoint defect by activating Rad53.

Effect of the *rfc5-1* mutation on *RNR3* expression. It has been shown that transcription of DNA damage-inducible genes requires a functional Rad53-dependent pathway (1). Since *RFC5* functions upstream of *RAD53*, we predicted that *RFC5* should be required for the transcriptional response to DNA damage. To test this point, *rfc5-1* mutants were examined for their ability to regulate the expression of the DNA damage-inducible gene *RNR3* in response to MMS. Cells grown at 25°C were shifted to 37°C for 2 h and then treated with MMS at 37°C for 3 h. As shown in Fig. 7A, the amount of *RNR3* mRNA was dramatically increased in response to MMS in wild-type cells. In contrast, *rfc5-1* mutants displayed a reduced ability to induce *RNR3* by MMS, although the basal level of *RNR3* mRNA in *rfc5-1* mutants was higher than in wild-type cells. These findings demonstrate that *RFC5* plays a key role in promoting DNA damage-induced transcription of the *RNR3* gene.

Since *TEL1* overexpression restored MMS-induced Rad53 modification in *rfc5-1* mutant cells (Fig. 6), we asked whether overexpression of *TEL1* rescues induction of *RNR3* transcription by MMS with the reporter plasmid pZZ13 (*RNR3-lacZ*) (36). *rfc5-1* mutants carrying pZZ13 were transformed with YEp plasmids containing *POL30*, *MEC1*, or *TEL1*. The transformants were treated with MMS at 37°C for 3 h and subjected to β-galactosidase assays (Fig. 7B). Overexpression of *TEL1* partially rescued the MMS-induced *RNR3* transcription in *rfc5-1* mutants, whereas overexpression of *POL30* or *MEC1* did not.

Effects of the *rfc5-1* mutation on mitotic entry and Rad53 activation in response to HU treatment. We have previously shown that *rfc5-1* mutants overexpressing *POL30* could grow at 37°C but they pursued mitosis following treatment with HU at 37°C (30). This result has raised the possibility that *rfc5-1* cells have defects in the S-phase checkpoint. To confirm this possibility, we examined the DNA content and spindle elongation in *rfc5-1* mutants when α-factor-arrested cells were released into HU at 37°C (Fig. 8). If cells are defective in the S-phase

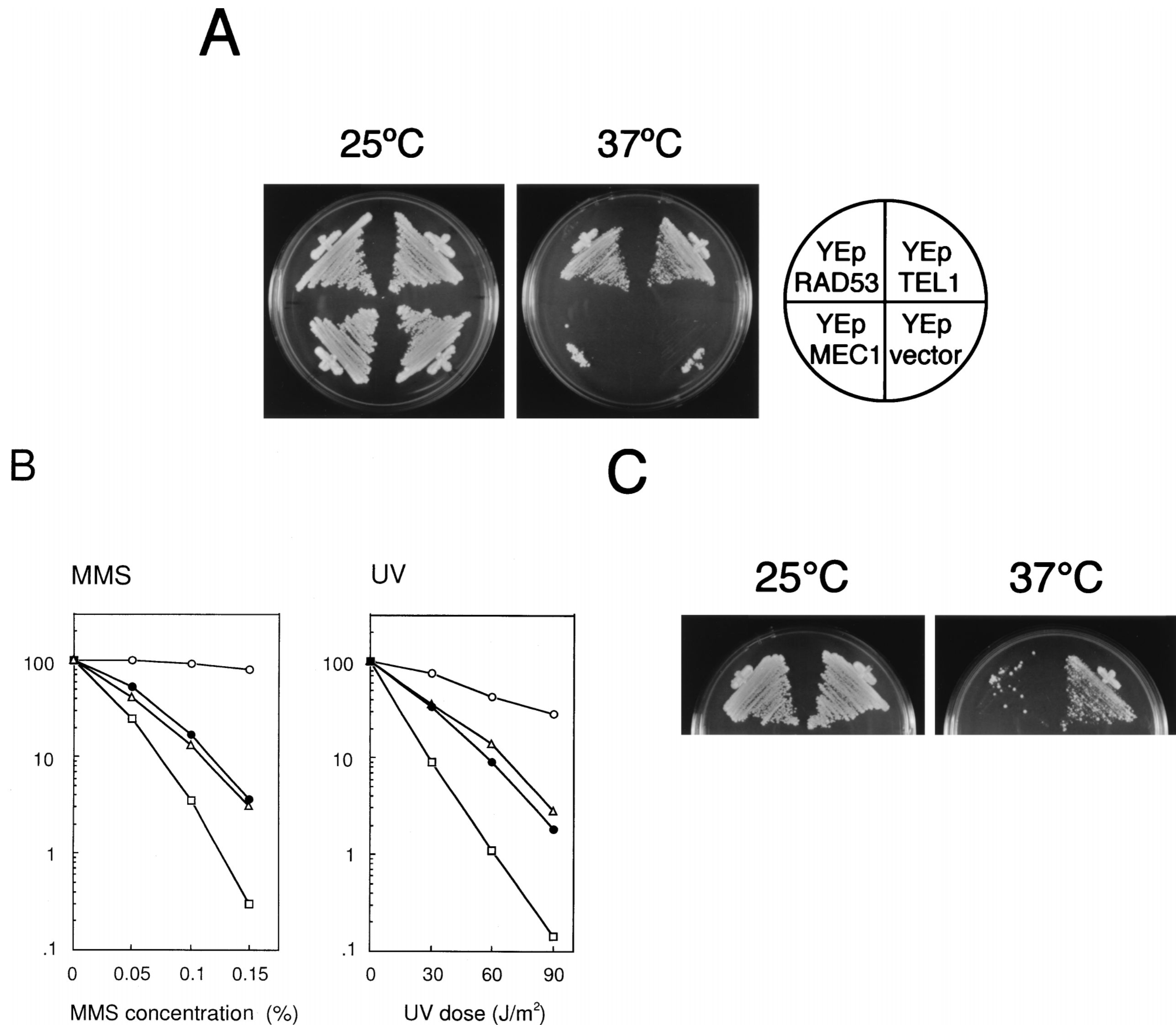


FIG. 5. Effects of *TEL1* overexpression on growth and DNA damage sensitivity in *rfc5* mutants. (A) The *rfc5-1* mutant (KSC766) cells transformed with different plasmids were streaked onto YEPD medium and incubated at 25 or 37°C. The plasmids transformed were YEpRAD53, YEpTEL1 (pDM198), YEpMEC1, and YEp vector (YEplac195). (B) The *rfc5-1* mutant (KSC766) cells carrying YEpT-POL30 were transformed with YCpRFC5 (○), YEpRAD53 (●), YEpTEL1 (pDM198) (△), or YEplac195 (□). The transformants in a log-phase culture at 37°C were treated with the indicated concentrations of MMS for 10 min or irradiated at the indicated doses with UV light. The viability of cells was estimated as described in Materials and Methods. (C) The *rfc5-1 tel1*Δ mutant (KSC1004) cells transformed with YEpRAD53 (left of plate) or YEpTEL1 (pDM198) (right of plate) were streaked onto YEPD medium and incubated at 25 or 37°C.

checkpoint, HU-treated cells should enter into mitosis, as evidenced by partial spindle elongation before completion of DNA replication. Wild-type and *rfc5-1* mutant cells completed mitosis within 100 min after release from G_1 in the absence of HU (data not shown). Flow-cytometric analysis showed that DNA replication was efficiently blocked in wild-type and *rfc5-1* mutant cells until 2 h after the release into HU (Fig. 8A). Under these conditions, most wild-type cells (97%) were arrested as large budded cells with short spindles and 98% of the cells remained viable 2 h after release. In contrast, 22% of *rfc5-1* mutant cells exhibited partially elongated spindles and 84% of the cells were inviable (Fig. 8B). These results support our previous results (30) indicating that the *rfc5-1* mutation is defective in the S-phase checkpoint in response to HU.

We next examined the replication block-induced activation of Rad53 in *rfc5* mutant cells. Although exposure of wild-type cells to HU or MMS at 30°C led to the modification of Rad53, HU produced a shift in Rad53-HA mobility that was less pronounced than that observed with MMS (data not shown). A similar result has been obtained by Sanchez et al. (23). Furthermore, modification of Rad53-HA in response to HU was greatly weakened at 37°C. It was therefore difficult to evaluate the effect of *rfc5-1* on HU-induced modification of Rad53. Since it has been shown that Rad53 phosphorylation correlates with increased kinase activity (27), we examined the kinase activity associated with Rad53 in response to HU treatment (Fig. 9). Rad53-dependent in vitro phosphorylation of Rad53 was detected in immune complexes from Rad53-HA-expressing cells. Rad53-HA protein became phosphorylated when immunoprecipitated and incubated in vitro with [γ-^{32}P]ATP. This property was used to demonstrate the kinase activity of Rad53. Only a low level of such protein kinase activity was observed in

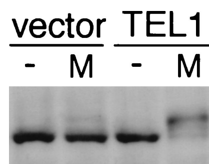


FIG. 6. Effects of *TEL1* overexpression on modification of Rad53 in *rfc5* mutants. The *rfc5-1* mutant (KSC766) cells were transformed with YCp-RAD53-HA and YEpT-TEL1 (TEL1) or pYO324 (vector). The transformants grown at 25°C were shifted to 37°C for 1 h and then incubated with YEPD (lanes −) or YEPD containing 0.1% MMS (lanes M) at 37°C for 2 h. The cells were subjected to Western blot analysis as described in Materials and Methods.

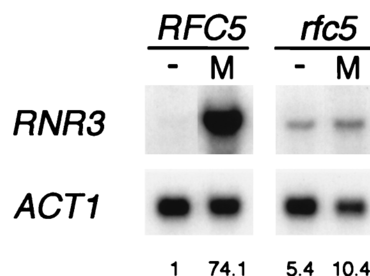
immune complexes from cells expressing a kinase-negative mutant version of Rad53 (Rad53-K227A-HA), in which the highly conserved lysine residue at position 227 was replaced by alanine (data not shown). It is therefore likely that the protein kinase activity observed in immune complexes is derived from a functional Rad53-HA protein kinase, although it remains possible that the kinase activity in HA immunoprecipitates is due to an associated protein kinase. Phosphorylation activity associated with Rad53-HA significantly increased when Rad53-HA was immunoprecipitated from wild-type cells that had been released from G_1 into HU. On the other hand, reduced kinase activity was observed in Rad53-HA immune complexes prepared from *rfc5-1* mutant cells after the release into HU (Fig. 9). These results suggest that *RFC5* is required for activation of Rad53 in the S-phase checkpoint pathway.

DISCUSSION

We have previously shown that the *rfc5-1* mutation is defective for both DNA replication and the S-phase checkpoint (30). At the restrictive temperature, *rfc5-1* mutants, besides being defective in DNA synthesis, enter mitosis with unevenly separated or fragmented chromosomes, resulting in a loss of viability. Overexpression of *POL30*, encoding PCNA, suppresses the ts growth defect of *rfc5-1* mutants but still allows *rfc5-1* cells in asynchronous culture to pursue mitosis following treatment with HU at the restrictive temperature (30). Thus, the role of Rfc5 in DNA replication and checkpoint control can be genetically separated. In this study, we confirmed that *rfc5-1* mutants have a checkpoint defect in response to replication block. When wild-type cells are synchronized in G_1 with α-factor and then released into HU, cells arrest within the S phase, the mitotic spindle does not elongate, and the cells remain arrested and viable (1). In contrast, when *rfc5-1* cells are released from G_1 synchrony into HU at the restrictive temperature, the cells arrest in mid-S phase but the mitotic spindle partially elongates, resulting in cell death. These results are consistent with mitotic entry without completion of DNA replication. There was an apparent uncoupling between the frequency of viability loss and mitotic spindle elongation in the HU-treated *rfc5-1* cells. A similar observation has been reported for *sad1*/*rad53* mutants (1). As discussed by Elledge (5), HU-induced lethality may result from events other than mitotic entry, such as alterations in nuclear architecture.

It has been proposed that active replication complexes generate a checkpoint control signal that inhibits the onset of mitosis during DNA replication. In support of this idea, Navas et al. (16, 17) have demonstrated that pol ε of *S. cerevisiae* serves not only as an essential replication enzyme but also as a potential sensor in the S-phase checkpoint. The evidence that Rfc5 plays a role in the S-phase checkpoint supports a direct link between the DNA replication machinery and the S-phase checkpoint. RFC is a structure-specific DNA-binding protein complex that recognizes the primer-template junction. RFC loads PCNA onto the primer terminus in an ATP-dependent reaction. pol δ and pol ε then bind to the DNA-RFC-PCNA complex to form a processive replication complex (2, 11, 32). These results suggest that Rfc5 and pol ε play a direct role in sensing the state of replication and transmitting this signal to the checkpoint machinery.

A



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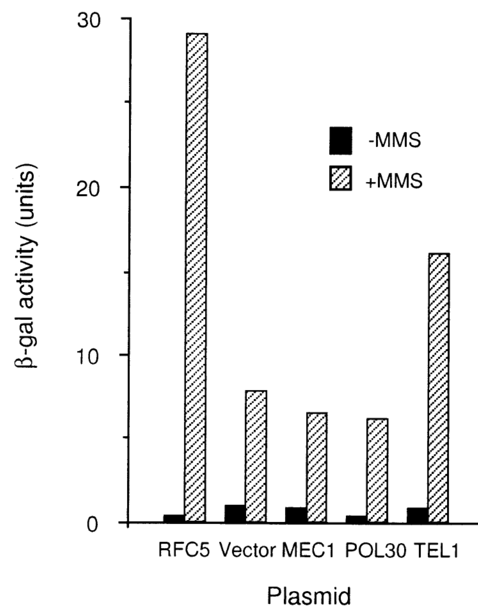
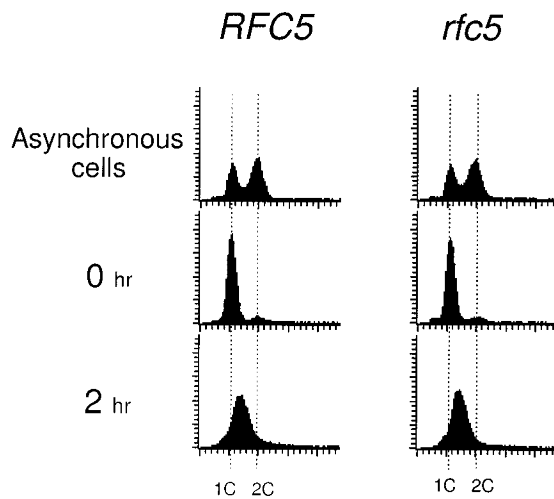


FIG. 7. *RNR3* expression in *rfc5* mutant cells. (A) *RFC5*$^+$ (KSC800) and *rfc5-1* mutant (KSC766) cells grown at 25°C were shifted to 37°C for 2 h and then incubated in the absence (lanes −) or the presence (lanes M) of 0.025% MMS at 37°C for 3 h. A 3-μg portion of total RNA prepared from the cells was separated on 1% formaldehyde–agarose gels and analyzed by Northern blot analysis with probes derived from *RNR3* and *ACT1*. The induction ratio of *RNR3* was determined by normalizing the autoradioactivities of the *RNR3* bands to those of the *ACT1* bands. (B) The *rfc5-1* mutant (KSC953) cells carrying the reporter plasmid pZZ13 (*RNR3-lacZ*) were transformed with indicated plasmids. The transformants grown at 25°C were shifted to 37°C for 2 h and incubated in the absence or presence of 0.02% MMS at 37°C for 3 h. The cells were then subjected to β-galactosidase (β-gal) assays. The values given (β-gal units) represent Miller units of at least two independent cultures for each transformant. The plasmids used were vector (YEplac195), POL30 (YEpPOL30), MEC1 (YEpMEC1), TEL1 (pDM198), and RFC5 (YCpRFC5).



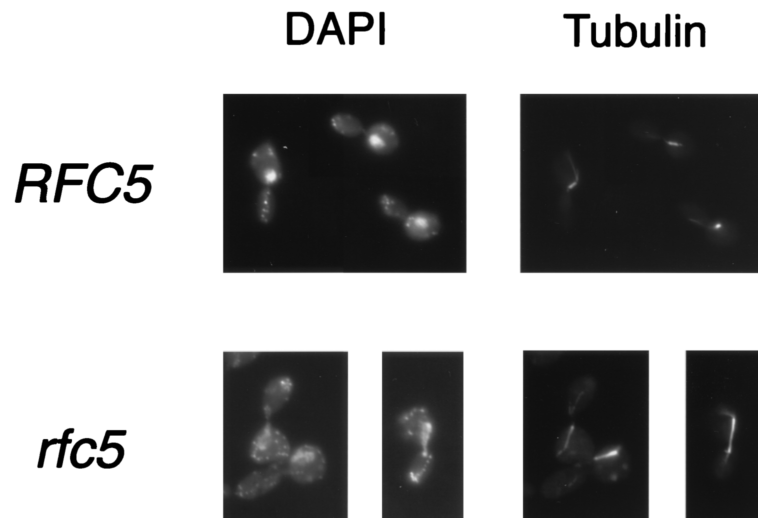


FIG. 8. Nuclear and spindle morphologies of *rfc5* mutants in the presence of HU. (A) DNA content by DNA flow cytometry of G_1-synchronized cells released into medium containing HU. *RFC5*$^+$ (KSC800) and *rfc5-1* mutant (KSC766) cells carrying YCp-RAD53-HA were synchronized in G_1 and released into YEPD containing 10 mg of HU per ml as described in Materials and Methods. Aliquots of cells were collected at the indicated times after release from α-factor treatment and examined for DNA content by flow cytometry. Dotted lines indicate the DNA content of 1C and 2C cells. The top panels represent asynchronous cells untreated with MMS at 25°C and are included as a reference. (B) Photomicrographs of *RFC5*$^+$ (KSC800) and *rfc5-1* mutant (KSC766) cells at 2 h after release from the G_1 block into medium containing HU. The cells were collected and fixed in formaldehyde. Nuclear and microtubular structures were visualized with 4′,6-diamidino-2-phenylindole (DAPI) and anti-tubulin antibodies, respectively. At least 200 cells were examined.

In this work, we showed that *rfc5-1* mutants are sensitive to DNA-damaging agents. DNA damage sensitivity can be due to different causes, such as defective DNA repair or defective checkpoint mechanisms. The checkpoint phenotype requires inappropriate cell cycle passage through the damaged stage. Consistent with the possibility that *rfc5-1* is defective in the DNA damage checkpoint, the MMS sensitivity of *rfc5-1* was partially suppressed by preventing the cell cycle progression with nocodazole treatment. The partial suppression by nocodazole block suggests that the *rfc5-1* mutation is also defective in DNA repair. In fact, the largest subunit of RFC, Cdc44, plays an important role in both DNA replication and DNA repair in vivo (14). Furthermore, we present evidence showing that *RFC5* is necessary for the induction of the repair machinery following DNA damage.

Paulovich and Hartwell have shown that a regulatory mechanism decreases the rate of S-phase progression in response to DNA damage (19). This control is dependent on the *MEC1* and *RAD53* checkpoint genes. We demonstrated that *RFC5* plays a role in regulating the S phase in response to DNA damage, although to a lesser extent than do *MEC1* and *RAD53*. The *rfc5-1* mutant gene caused cells to progress through the S phase more rapidly in the presence of MMS than did the wild-type *RFC5* gene. However, in the presence of MMS, *rfc5-1* mutants passed through replication more slowly than they did in the absence of MMS. In contrast, *mec1* and *rad53* mutants

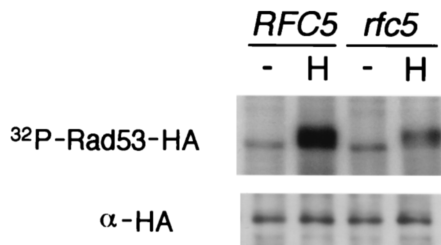


FIG. 9. Rad53 kinase activity of *rfc5* mutants in the presence of HU. $RFC5^+$ (KSC800) and *rfc5-1* mutant (KSC766) cells carrying YCp-RAD53-HA were collected either after synchronization in G_1 (lanes −) or 2 h after release from the G_1 block into medium containing HU (lanes H). Rad53-HA was immunoprecipitated, and in vitro kinase assays were performed as described in Materials and Methods (top). Equal portions of the immunoprecipitates were subjected to immunodetection of Rad53-HA to quantify the amount of Rad53-HA immunoprecipitated (bottom). For the purpose of quantitation, the SDS-PAGE was run for a short time to immunodetect Rad53. Note that the autophosphorylation affected only the activated material and caused a further smeared shift.

progress rapidly through the S phase regardless of whether MMS is present. The partial defect in the S-phase regulation in *rfc5-1*, as compared to the complete defect in *mec1* and *rad53*, suggests the following possibilities. (i) *rfc5-1* mutants may still have some residual checkpoint activity at the restrictive temperature due to the leakiness of the conditional mutation. (ii) *RFC5* may have functions that are partially redundant with other checkpoint genes in controlling S-phase progression. Most recently, Paulovich et al. (20) showed that *RAD9*, *RAD17*, and *RAD24* are also involved in controlling the S-phase rate, although to a lesser extent than *MEC1* and *RAD53*. Interestingly, the *RAD24* gene encodes a protein related to RFC (7), raising the possibility that *RFC5* and *RAD24* share overlapping functions to activate the *MEC1*- and *RAD53*-dependent pathway in the S-phase regulation.

DNA damage checkpoint pathways also prevent cells in the G_2 phase from undergoing mitosis and those in G_1 from entering the S phase. We examined *rfc5-1* mutant cells for their ability to phosphorylate Rad53 in response to DNA damage at G_2/M. In contrast to MMS treatment during the S phase, MMS treatment of nocodazole-arrested *rfc5* cells still induced the modification of Rad53. The observation that Rad53 is modified in response to DNA damage in G_2/M-arrested *rfc5-1* cells indicates that there are other checkpoint components that transduce the signal to Rad53 in the G_2/M checkpoint. Consistent with this possibility, MMS treatment is unable to induce Rad53 phosphorylation in nocodazole-arrested *mec3* mutant cells (31). Interestingly, Navas et al. (16, 17) have shown that *pol2* mutants are defective in transducing DNA damage signal in S phase but are proficient for G_1 and G_2 DNA damage checkpoints. RFC plays a role in loading pol ε onto the replicating DNA template in the S phase. These results suggest that Rfc5 and Pol2 may play a cooperative role in DNA damage checkpoints during S phase.

The checkpoint pathway is considered to be regulated by at least three components: a monitoring system to detect the change in DNA structure, a signal pathway to transmit the information from this monitoring system to the cell cycle machinery, and a target that the signal acts upon to regulate the cell cycle. The Rad53 protein kinase is a signal transducer in DNA damage and replication checkpoint pathways and is also needed for the transcriptional response to DNA damage (1, 35). Rad53 is phosphorylated in response to DNA damage and replication block (23, 31). Several lines of evidence presented here strongly argue that *RFC5* functions upstream of *RAD53* in the signaling pathway. First, overexpression of *RAD53* suppressed the DNA damage sensitivity of *rfc5-1*. Second, phosphorylation of Rad53 in response to MMS treatment was reduced in *rfc5-1* mutants. Third, MMS-induced expression of *RNR3* was reduced in *rfc5-1* mutants. A simple explanation for these results is that *RFC5* functions upstream of *RAD53* in the same pathway and that overexpression of *RAD53* would enhance its role in the checkpoint pathway, resulting in a reduced requirement for upstream gene function. We assume that in this model, Rfc5 plays a sensory role or a signal transduction role in the pathway leading to the activation of Rad53. Recently, Lydall and Weinert (13) have provided evidence that damage processing is necessary for activating the DNA damage checkpoint. It is therefore possible that DNA damage is incorrectly processed in *rfc5-1* mutants, so that it is not well recognized by the Rad53-dependent checkpoint control.

Phosphorylation of Rad53 in response to DNA damage and replication block is controlled by Mec1 and Tel1, which belong to the ATM-related kinase family (15, 24). *TEL1* has functions that are partially redundant with *MEC1* in response to DNA damage and replication block. Although *tel1*Δ single mutants themselves have no known checkpoint defect, the *tel1*Δ mutation shows several genetic interactions with the *mec1* mutation (15, 23). Furthermore, overexpression of *RAD53* suppresses the *mec1* mutation. These results indicate that Mec1 and Tel1 function upstream of Rad53 and may directly phosphorylate and activate Rad53. Overexpression of *TEL1* partially suppressed the DNA damage sensitivity of *rfc5-1* mutants, restoring the phosphorylation of Rad53 and transcriptional activation of *RNR3* in response to MMS in *rfc5-1* mutants. Thus, *TEL1* overexpression appears to suppress the *rfc5-1* checkpoint defect by activating Rad53. In contrast, overexpression of *MEC1* failed to suppress the *rfc5-1* mutation. Furthermore, *RAD53* overexpression was unable to suppress the *rfc5-1* mutation in the absence of *TEL1*. One explanation for these results is that *TEL1* and *MEC1* function downstream of *RFC5* in a linear pathway. If so, the Mec1 function might be tightly regulated by the upstream signal through Rfc5 whereas the Tel1 function might not. Alternatively, it is possible that *TEL1* acts in a parallel pathway to *RFC5*. In this case, Rfc5 may generate a signal detected by Mec1 and relayed to Rad53, whereas Tel1 may respond to a different signal and feed the signal to activate Rad53 independently of the Rfc5-dependent pathway.

It remains unclear how overexpression of *TEL1* or *RAD53* suppresses the growth defect of *rfc5-1*. Since *TEL1* overexpression restored Rad53 modification and *RNR3* transcriptional induction in response to MMS in *rfc5-1* mutants, *TEL1* or *RAD53* could rescue the ts growth defect of *rfc5-1* through the checkpoint control. It is well known that checkpoint-defective mutations decrease the restrictive temperature of mutants defective in DNA replication (33). If this is the case for the *rfc5-1* mutation, rescue of the checkpoint defect should allow *rfc5-1* mutants to grow at the restrictive temperature. An alternative possibility is that overexpression of *TEL1* or *RAD53* suppresses the growth defect of *rfc5-1* through the role of *RAD53* in DNA replication, because Rad53 is considered to have an essential function associated with DNA replication. Further studies are required to establish firmly the order of relative functions of *RAD53* and *RFC5* in the checkpoint control and DNA replication.

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