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Rfc5, a small subunit of replication factor C complex, couples DNA replication and mitosis in budding yeast

(checkpoint control/*SPK1/MEC2/RAD53/SAD1*/proliferating cell nuclear antigen)

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ABSTRACT **The inhibition of DNA synthesis prevents mitotic entry through the action of the S phase checkpoint. In the yeast *Saccharomyces cerevisiae*, an essential protein kinase, Spk1/Mec2/Rad53/Sad1, controls the coupling of S phase to mitosis. In an attempt to identify genes that genetically interact with Spk1, we have isolated a temperature-sensitive mutation, *rfc5-1*, that can be suppressed by overexpression of *SPK1*. The *RFC5* gene encodes a small subunit of replication factor C complex. At the restrictive temperature, *rfc5-1* mutant cells entered mitosis with unevenly separated or fragmented chromosomes, resulting in loss of viability. Thus, the *rfc5* mutation defective for DNA replication is also impaired in the S phase checkpoint. Overexpression of *POL30*, which encodes the proliferating cell nuclear antigen, suppressed the replication defect of the *rfc5* mutant but not its checkpoint defect. Taken together, these results suggested that replication factor C has a direct role in sensing the state of DNA replication and transmitting the signal to the checkpoint machinery.**

The eukaryotic mitotic cell cycle consists of a temporally ordered series of events in which the initiation of late events is dependent on the completion of the early ones. This order is maintained by mechanisms called checkpoint controls that monitor completion of earlier events and control cell cycle progression (1–3). In eukaryotes, for example, incomplete DNA replication or DNA damage induces cell cycle arrest in G_2 before mitosis. This dependency or checkpoint is important for ensuring that the cell does not divide unless the chromosomes have been completely duplicated.

The budding yeast *Saccharomyces cerevisiae* has been one of the best model systems for the identification and genetic analysis of the cell cycle. In *S. cerevisiae*, a number of genes, such as *RAD9*, *RAD17*, *RAD24*, *MEC1/ESR1*, *SPK1/MEC2/RAD53/SAD1*, and *MEC3*, involved in the DNA damage checkpoint have been identified (4–7). *MEC1* and *SPK1* are also necessary to inhibit the onset of mitosis in response to incomplete replication (6, 7). *MEC1* is a homolog of the human *ATM* gene, which is mutated in patients with ataxia telangiectasia (8). *SPK1* encodes a dual-specificity protein kinase (9). The mechanism that prevents mitosis until completion of S phase remains a central unanswered question.

Active replication complexes have been proposed as potential sources of the S phase signal because DNA replication must be completed before initiation of mitosis (3). In support of this idea, Navas *et al.* (10) have demonstrated that DNA polymerase (pol) ε of *S. cerevisiae* serves not only as an essential replication enzyme but also as a sensor in the S phase checkpoint, which is consistent with a role for active replication complexes in this process. However, it is not yet known how the replication apparatus senses the unreplicated DNA and sends the signal to the checkpoint machinery.

To identify genes that genetically interact with Spk1, we have isolated temperature-sensitive (ts^-) mutants that can be suppressed by high dosages of *SPK1*. We identified a mutation of *RFC5* that encodes a small subunit of replication factor C (RFC) complex. In this paper, we present evidence that Rfc5 has a dual role in DNA replication and S phase checkpoint, demonstrating a direct link of the DNA replication machinery to the S phase checkpoint.

MATERIALS AND METHODS

General Methods. DNA was manipulated by standard procedures (11). Standard genetic techniques were used for manipulating yeast strains (12). To construct isogenic wild-type and *rfc5-1* strains, YIplac128 plasmid bearing a *Sal*I-*Hin*dIII fragment of the wild-type *RFC5* was homologously integrated into the chromosome of *rfc5-1* strain (KSC766) to create the isogenic $RFC5^+$ strain (KSC800).

Mutant Isolation. A *spk1-101* [previously named as *hys1-1* (13)] strain carrying a wild-type *SPK1* gene on a high copy plasmid (YEpSPK1) was mutagenized with ethylmethane sulfonate. Out of ≈10,000 colonies, five colonies that grew at 37°C in the presence of YEpSPK1, but not in the absence of YEpSPK1, were obtained. Two classes of mutations were identified. The first class comprises a synthetic ts^- mutation with *spk1-101* in the absence of YEpSPK1. The second class comprises a ts^- mutation that could be suppressed by overexpression of *SPK1*. Five different complementation groups were identified: four groups belong to the first class and the remaining group (*rfc5-1*) belongs to the second class.

Cloning of the *RFC5* Gene. Strain KSC766 (*rfc5-1 ura3*) was transformed with a yeast genomic library constructed on YCp50 and transformants were selected for restoration of growth at 37°C. Nucleotide sequences were determined by the dideoxy chain termination method. *RFC5* corresponds to the open reading frame *YBR0810* on chromosome II (GenBank accession no. X78993).

Localization of the Mutation Site. To recover the mutation allele, genomic DNA prepared from KSC800 was digested with either *Sal*I or *Hin*dIII, self-ligated, and transformed into *Escherichia coli*. Subcloning and complementation analyses indicated that the *Hin*dIII-*Mlu*I fragment of the *rfc5-1* gene contains the mutation. A single mutation site was determined by sequence analysis of the region.

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Abbreviations: ts^-, temperature sensitive; RFC, replication factor C; HA, hemagglutinin, HU, hydroxyurea; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; YPD medium, yeast extract/peptone/dextrose medium.

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Construction of Hemagglutinin (HA)-Tagged *RFC5*. The DNA sequences encoding the epitope recognized by the anti-HA monoclonal antibody 12CA5 were attached in-frame to the C-terminal end of *RFC5* using polymerase chain reaction. When expressed from its own promoter on YCp plasmid (pR5H), the tagged construct (*RFC5*-HA) was found to fully complement a null mutation (*rfc5Δ::LEU2*) in *RFC5*.

Purification of RFC. RFC activity from *rfc5Δ::LEU2* cells carrying plasmid pR5H (*RFC5*-HA) was purified by Blue-Sepharose and subsequent single-stranded DNA cellulose columns and applied to MonoQ column as described previously (14).

Other Methods. Pulsed-field gel electrophoresis, fluorescence-activated cell sorting analysis, and immunofluorescence microscopy analysis were performed as described previously (13).

RESULTS

Isolation of the *rfc5* Mutation. In a search for elements that might be involved in checkpoint control by Spk1, we carried out a screen to isolate ts⁻ mutants that were suppressed by overexpression of *SPK1*. One recessive mutation, *rfc5-1* (see below), was identified (Fig. 1*A*). *RFC5* was cloned by complementation of the ts⁻ phenotype and shown to be the authentic gene by homologous integration. The predicted amino acid sequence of the *RFC5* gene shows an extensive similarity to the subunits of yeast and human RFC, with the highest similarity to human RFC38 (Fig. 1*B*) (14–19). RFC is a multiprotein complex consisting of one large and four small subunits that is required for processive DNA synthesis by pols δ and ε (20–22). *CDC44* encodes the largest subunit (15, 16) and *RFC2*, *RFC3* and *RFC4* encode the small subunits (14, 17, 18). Therefore, the gene and the mutation will be referred to as *RFC5* and *rfc5-1*, respectively.

The position of the mutation in *rfc5-1* was determined and shown to contain a single nucleotide change (from G to A) at base 128, which results in a change from Gly to Glu at amino acid position 43 (Fig. 1*B*). This mutation is in the most highly conserved motif, **Gly**XXGlyXGlyLys, of ATP/GTP binding proteins. One allele of the *RFC5* gene was disrupted with the *LEU2* gene in diploid cells. When they were sporulated, tetrads segregated at a ratio of 2:2 viable/nonviable. All viable segregants were Leu⁻, consistent with the *RFC5* gene product being essential for growth.

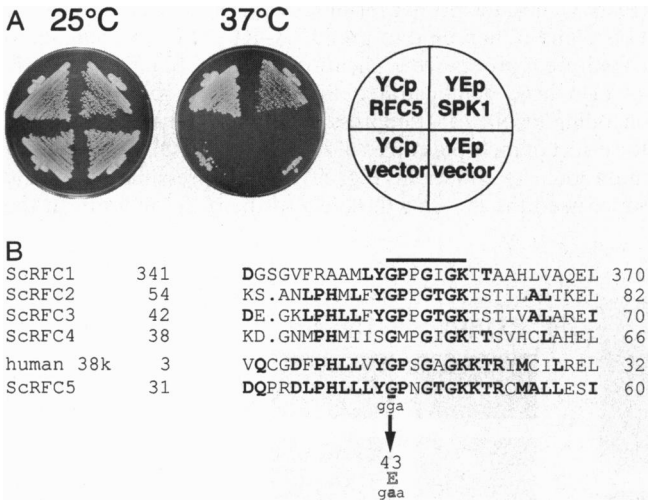

FIG. 1. Isolation of the *RFC5* gene. (*A*) Suppression of the *rfc5* mutant by *SPK1*. The *rfc5-1* mutant cells transformed with different plasmids were streaked onto yeast extract/peptone/dextrose (YPD) medium and incubated at 25°C or 37°C. Plasmids were as follows: YEplac195 (YEp vector), YEpSPK1, YCplac33 (YCp vector), and YCpRFC5. (*B*) A single amino acid substitution conferred by the *rfc5-1* mutation. DNA and amino acid sequences of wild-type and *rfc5-1* reveal substitution of guanosine by adenosine at base 128, converting a glycine to a glutamate codon at amino acid residue 43. Around the mutation site, alignment of the amino acid sequences of Rfc5, yeast RFC subunits, and human RFC38 are also shown. The identical amino acids with those of Rfc5 are in boldface type. The putative ATP/GTP binding motif is lined. Cdc44 is shown as ScRfc1.

Rfc5 Is a Component of RFC Complex. To demonstrate that Rfc5 is a component of RFC complex, RFC was partially purified by column chromatography and the fractions were subjected to Western blot analysis (Fig. 2). The Rfc5 protein coeluted with RFC activity and the Rfc2 protein from three successive columns, blue Sepharose, single-stranded DNA cellulose, and MonoQ column. These results strongly suggest that Rfc5 is a component of yeast RFC complex. Consistent with our results, Cullmann *et al.* (23) recently identified Rfc5 as a subunit of the yeast RFC.

Effect of the *rfc5-1* Mutation On the Cell Cycle. Spk1 plays an active role in preventing nuclear division until S phase is completed. We therefore examined whether the *rfc5* mutation affected the S phase checkpoint. An S phase checkpoint deficiency should cause rapid loss of cell viability, because it enters mitosis with incompletely replicated DNA. To examine the terminal phenotype of *rfc5-1* cells in the cell cycle, exponentially growing *rfc5-1* cells at 25°C were transferred to 37°C. Aliquots were collected to determine cell viability, DNA content, and nuclear and spindle morphologies. The number of *rfc5-1* cells increased about 4-fold until the cells arrested. After 6 hr at 37°C, ≈67% of *rfc5* cells arrested as large-budded cells and the cell viability rapidly dropped to 0.1% (Fig. 3). This lethality was rescued by α-factor treatment (G_1 arrest), but not by hydroxyurea (HU; DNA synthesis inhibitor) treatment (Fig. 3*A*). Thus, the loss of cell viability in *rfc5* is correlated with the S phase. Fluorescence-activated cell sorter analysis revealed that the *rfc5-1* mutant strain arrested with a S-to-G_2/M phase DNA content (Fig. 3*B*), suggesting that defects occur during the progression from S to mitosis in *rfc5-1* cells.

To obtain information about the state of chromosomal DNA in *rfc5* cells at the restrictive temperature, chromosomes were analyzed by pulsed-field gel electrophoresis (Fig. 3*C*). In this assay, only fully replicated DNA enters the gel and migrates properly (24). In control experiments, DNA of *rfc5-1* cells treated with HU at 25°C failed to enter the gel. DNA prepared from *rfc5-1* cells at 37°C entered the gel with greatly reduced

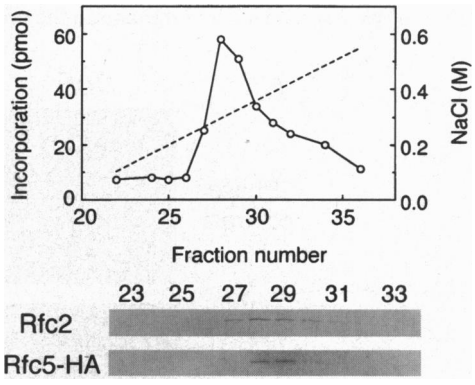

FIG. 2. Copurification of Rfc5 with RFC activity and Rfc2. RFC activity from *rfc5Δ::LEU2* cells carrying plasmid pR5H (*RFC5*-HA) was purified as described. Each fraction of MonoQ column chromatography was subjected to assay for RFC activity (14) and Western blot analysis with rabbit antiserum against Rfc2 protein or with the anti-HA monoclonal antibody 12CA5. Rfc2 and HA-tagged Rfc5 proteins were visualized by ProtBlot Western Blot AP system (Promega) and the Enhanced Chemiluminescence kit (Amersham), respectively. Open circles represent RFC activity. The dotted line indicates the concentration of NaCl in elution buffer.

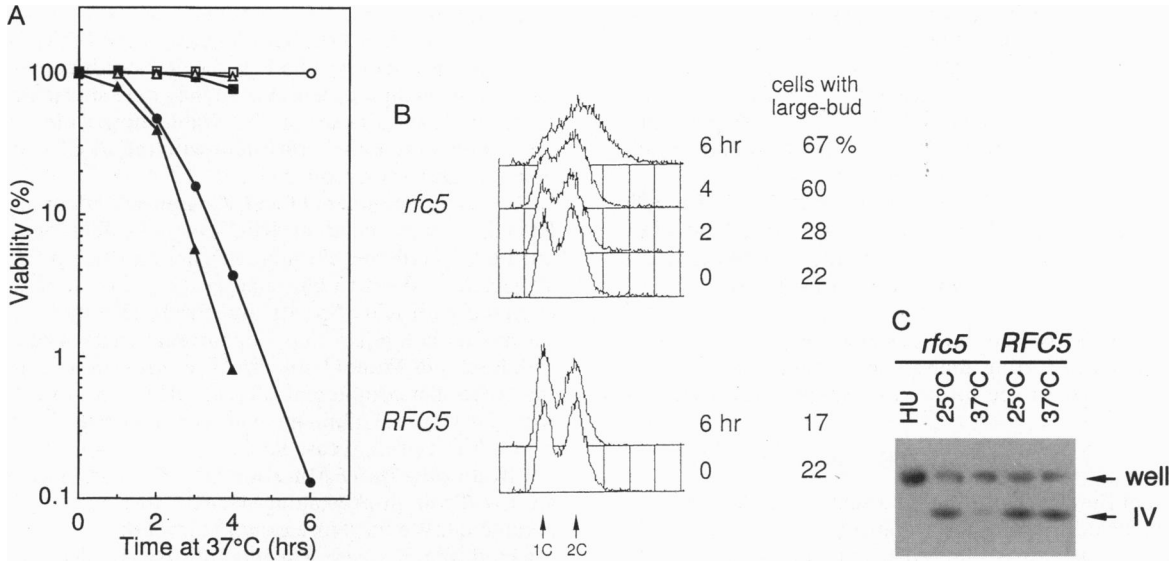

FIG. 3. Growth properties of the *rfc5-1* mutant. (*A*) Cell viability. Wild-type (○) and *rfc5-1* (●) cells grown exponentially at 25°C were shifted to 37°C. For treatment with α-factor and HU, log-phase cultures were initially treated with α-factor at 4 μg/ml for 2 hr at 25°C. Samples were washed with YPD, split, and resuspended in YPD containing α-factor at 4 μg/ml (wild type, □; *rfc5-1*, ■) or 10 mg/ml HU (wild type, △; *rfc5-1*, ▲). The culture containing α-factor was incubated at 37°C and α-factor was subsequently added every 2 hr. The other culture containing HU was further incubated at 25°C for 2 hr and then shifted to 37°C. The viability of cells was estimated as described (5). (*B*) DNA content analyzed by flow cytometry. The DNA content of wild-type and *rfc5-1* cells incubated at 37°C for 0–6 hr was analyzed by a Becton Dickinson FACScan. Positions of cells with 1C and 2C DNA are shown by arrows. The percentage of large-budded cells was determined microscopically. (*C*) Pulsed-field gel electrophoresis of chromosomal DNA. Wild-type and *rfc5-1* cells were grown at 25°C and then transferred to 37°C for 4 hr. For HU treatment, *rfc5-1* cells were incubated in HU (10 mg/ml)-containing medium at 25°C for 4 hr. The top row of bands indicates where the samples were loaded (well) and contains residual materials that were not able to migrate into the gel. IV denotes bands of chromosome IV.

efficiency relative to wild-type DNA. These results indicate that *rfc5-1* cells do not complete DNA replication at the restrictive temperature, consistent with the involvement of Rfc5 in DNA replication.

Nuclear and tubulin morphologies in *rfc5* cells were abnormal at the restrictive temperature (Fig. 4). Cells with unequal segregation of DNA between mother and daughter cells or with fragmented or streaked nuclear DNA were frequently seen after 4 hr at 37°C; ≈29% of *rfc5* cells displayed these morphologies at 6 hr (Fig. 4*A*). Immunofluorescence microscopy in analysis with anti-tubulin antibody revealed cells containing elongated spindles (Fig. 4*B*), consistent with mitotic entry with incompletely replicated DNA. Furthermore, a fraction of cells contained abnormal spindles (Fig. 4*B*) as observed in *mec2* mutants in the presence of HU (6). These data suggest that the *rfc5-1* mutation is defective in the S phase checkpoint.

Effect of *POL30* Overexpression On the *rfc5* Mutant. RFC is a multisubunit DNA-activated ATPase that binds to the primer-template junction (20–23). The primary biochemical function of RFC is to load the proliferating cell nuclear antigen (PCNA) onto the primer terminus with the hydrolysis of ATP. Pols δ and ε then bind to the DNA–RFC–PCNA complex to constitute a processive replication complex. Since McAlear *et al.* (16) have demonstrated that multiple copies of *POL30* encoding PCNA (25) suppress *cdc44* mutations, we examined the effect of multiple copies of *POL30* (YEpPOL30) in the *rfc5* mutation. As shown in Fig. 5*A*, overexpression of *POL30* suppressed the ts⁻ growth defect of the *rfc5-1* mutant. In the

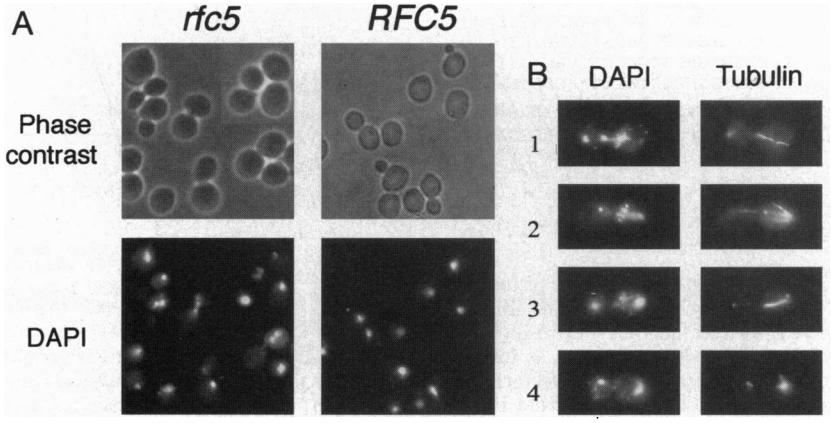

FIG. 4. Nuclear and spindle morphologies. (*A*) Photographs of DAPI stained *rfc5-1* cells. Wild-type and *rfc5-1* cells grown at 25°C were shifted to 37°C for 6 hr. Cells were fixed in ethanol and examined by phase contrast microscopy for 4′,6-diamidino-2-phenylindole (DAPI) staining. (*B*) Nuclear distribution and microtubular structures in *rfc5-1* cells. Cells 1 and 2 are large-budded cells with fragmented nuclear DNA; cells 3 and 4 are large-budded cells with unequal segregation of DNA between mother and daughter cells. *rfc5-1* cells grown at 25°C were shifted to 37°C for 6 hr and then fixed in formaldehyde. Nuclear morphology was visualized with DAPI and microtuble morphology was visualized with antitubulin antibodies.

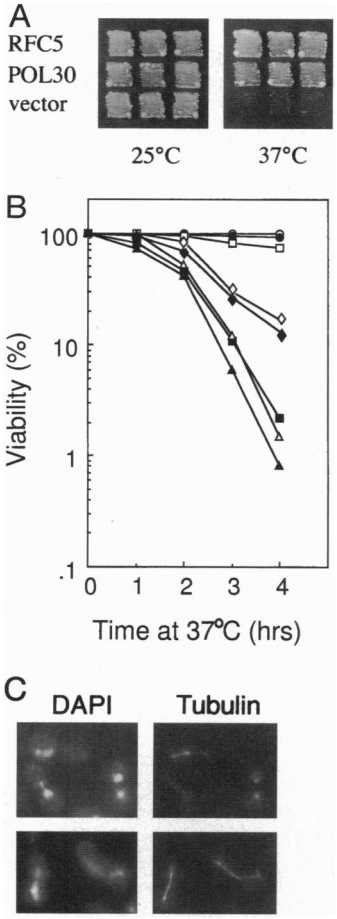

FIG. 5. Effect of overexpression of *POL30* on the *rfc5* mutation. (*A*) Suppression of the *rfc5* mutant by *POL30*. The *rfc5-1* mutant cells transformed with different plasmids were streaked onto YPD medium and incubated at 25°C or 37°C. Plasmids were as follows: top row, YCpRFC5; middle row, YEpPOL30; bottom row, YEplac195 (vector). (*B*) HU lethality. *rfc5-1* cells carrying YCpRFC5 (○, ●), YEplac195 (△, ▲), YEpPOL30 (□, ■), or YEpSPK1 (◇, ◆) were grown to log phase at 25°C. The cultures were divided into two portions. Cells in one part (open symbols) were shifted to 37°C. HU (10 mg/ml) was added into the other part (solid symbols). The culture was further incubated at 25°C for 1.5 hr and then shifted to 37°C. Viability of cells was estimated as described (5). (*C*) Nuclear distribution and microtubular structures. *rfc5-1* cells carrying YEpPOL30 grown at 25°C were treated with HU at 10 mg/ml for 1.5 hr, shifted to 37°C for 4 hr, and then fixed in formaldehyde. Nuclear and microtuble morphologies were visualized as described in the legend of Fig. 4*B*.

presence of HU, *rfc5-1* mutant cells carrying YEpPOL30 lost viability rapidly at 37°C, whereas YEpSPK1 partially rescued the viability loss (Fig. 5*B*). From the nuclear and tubulin morphologies, the HU-arrested *rfc5-1* cells with YEpPOL30 appeared to enter mitosis (Fig. 5*C*). Thus, overexpression of PCNA suppresses the DNA replication defect associated with the *rfc5-1* mutation but fails to suppress its S phase checkpoint defect. These results support the idea that Rfc5 has a dual role in S phase; it is required for DNA replication and generates a signal that inhibits the onset of mitosis.

DISCUSSION

It has been proposed that active replication complexes generate a checkpoint control signal that inhibits the onset of mitosis during DNA replication. The results presented here suggest that one of the RFC subunits, Rfc5, has a direct role in sensing incomplete DNA replication and transmitting the signal to the checkpoint machinery. The biochemical function of RFC has been elucidated in yeast and mammalian cells (18–20). 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. Pols δ and ε then bind to the DNA–RFC–PCNA complex to constitute a processive replication complex. Consistent with the biochemical function, overexpression of PCNA suppressed the replication defect of the *rfc5* mutant but not its checkpoint defect. Interestingly, some conditional lethal alleles in pol ε are defective in the S phase checkpoint (10). The pol ε and Rfc5 may have a direct role in sensing the state of replication and transmitting the signal to the checkpoint machinery. The structural and functional properties of RFC are highly conserved from yeast to human. This raises an intriguing possibility that human RFC38 and its homologues may be involved in the S phase checkpoint control in mammalian cells.

RFC is a multiprotein complex consisting of one large and four small subunits. Even though the five RFC subunits share high sequence similarity, they are all essential for cell viability. This indicates that the subunits of RFC complex are not functionally redundant, instead each subunit may carry out different essential role(s) in the RFC complex. Consistent with this possibility, the *rfc5* mutant was defective in DNA replication and entered into mitosis, whereas *cdc44* mutants defective in the largest subunit of RFC complex arrest at G_2/M phase and do not enter into mitosis (15, 16). Therefore, the Rfc5 subunit has a role in the S phase checkpoint function, whereas the Cdc44 subunit may not. The structural features could explain the unique role of Rfc5. The *rfc5-1* mutation contains an alteration of the glycine residue within the conserved nucleotide binding site, **Gly**XXGlyXGlyLys, of ATP/GTP binding proteins; in $p21^{ras}$, this region is involved in binding phosphate groups of the nucleotide (26). Although the *rfc5-1* mutation is likely to affect the ATPase activity of Rfc5, its ATPase activity may not be essential for the function of RFC because the *rfc5-1* mutant can grow at lower temperature. The domain around the nucleotide binding site in Rfc5 is slightly divergent from that of other RFC subunits, suggesting that this domain may be the defining feature unique to the Rfc5 protein. Supporting this idea, an analogous mutation in *RFC2* resulted in lethality at any temperatures (unpublished data).

It is likely that some components are shared in the DNA damage and the S phase checkpoint pathways (1–3). At this moment, the question whether the *rfc5-1* mutation is defective in the DNA damage checkpoint remains unanswered. At 37°C, *rfc5-1* mutant cells overexpressing PCNA were extremely sensitive to DNA-damaging agents, such as methylmethane sulfonate and UV irradiation (data not shown). The sensitivity to DNA-damaging agents may be attributable to a failure to cell cycle arrest, although we have not ruled out a direct role of Rfc5 in DNA repair.

The identification of Rfc5 as part of a potential checkpoint sensor will focus attention upon precisely what physical structures are being sensed through Rfc5 and how Rfc5 transmits this signal to other members of the checkpoint apparatus. If Rfc5 is a sensor, one possibility is that Rfc5 senses the functionality or balances in the activities of RFC components. In this regard, it will be of interest to test whether other subunits of RFC complex are also involved in the checkpoint sensing of incomplete DNA replication. Spk1 protein kinase has a checkpoint function, possibly acting as a positive regulator of DNA replication and as a negative regulator of mitosis. Overexpression of *SPK1* is capable of suppressing the *rfc5-1* mutant but not the lethality caused by the *rfc5* disruption. Furthermore, overexpression of *SPK1* failed to suppress the *cdc44-1* mutant (data not shown). These results suggest that Rfc5 may sense the state of DNA replication and feed signals into a central Spk1 kinase conduit that controls the cell cycle. Consistent with this hypothesis, kinase activity of Spk1 in the

rfc5-1 mutant was found to be less stimulated by HU treatment than that in the wild-type strain (unpublished data).

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