Molecular Biology of the Cell
Vol. 10, 3991–4003, December 1999

Replication Factor C3 of *Schizosaccharomyces pombe*, a Small Subunit of Replication Factor C Complex, Plays a Role in Both Replication and Damage Checkpoints

Midori Shimada,*† Daisuke Okuzaki,* Seiji Tanaka,* Takahiro Tougan,* Katsuyuki K. Tamai,*† Chikashi Shimoda,† and Hiroshi Nojima*‡

*Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; and †Department of Biology, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan

Submitted April 5, 1999; Accepted September 8, 1999
Monitoring Editor: Mitsuhiro Yanagida

We report here the isolation and functional analysis of the *rfc3*$^+$ gene of *Schizosaccharomyces pombe*, which encodes the third subunit of replication factor C (RFC3). Because the *rfc3*$^+$ gene was essential for growth, we isolated temperature-sensitive mutants. One of the mutants, *rfc3-1*, showed aberrant mitosis with fragmented or unevenly separated chromosomes at the restrictive temperature. In this mutant protein, arginine 216 was replaced by tryptophan. Pulsed-field gel electrophoresis suggested that *rfc3-1* cells had defects in DNA replication. *rfc3-1* cells were sensitive to hydroxyurea, methanesulfonate (MMS), and gamma and UV irradiation even at the permissive temperature, and the viabilities after these treatments were decreased. Using cells synchronized in early G2 by centrifugal elutriation, we found that the replication checkpoint triggered by hydroxyurea and the DNA damage checkpoint caused by MMS and gamma irradiation were impaired in *rfc3-1* cells. Association of Rfc3 and Rad17 in vivo and a significant reduction of the phosphorylated form of Chk1 in *rfc3-1* cells after treatments with MMS and gamma or UV irradiation suggested that the checkpoint signal emitted by Rfc3 is linked to the downstream checkpoint machinery via Rad17 and Chk1. From these results, we conclude that *rfc3*$^+$ is required not only for DNA replication but also for replication and damage checkpoint controls, probably functioning as a checkpoint sensor.

INTRODUCTION

The eukaryotic cell cycle is a complex mechanism requiring the coordination of many different macromolecular processes, including synthesis of molecular components and their assembly and disassembly as required to fulfill their functions. Along with the machinery that regulates cell cycle progression, cells are equipped with surveillance mechanisms known as checkpoints, which ensure that all processes in one cell cycle phase are completed before the next phase begins (Hartwell and Weinert, 1989). Checkpoint mechanisms induce cell cycle arrest at specific points when DNA or other cellular components are damaged and maintain the arrrested state until signals indicating recovery from the damage are received, to minimize somatic genetic alterations. If checkpoint mechanisms are impaired, cells proceed to a catastrophic and lethal attempt to segregate unreplicated chromosomes even if the cell cycle is perturbed by blockage of DNA replication or by unrepaired DNA damage.

Yeast is one of the best-studied model systems for the identification and functional analysis of checkpoints by genetic and biochemical approaches. Among the best characterized are a DNA damage checkpoint triggered by UV or gamma irradiation and a DNA replication checkpoint triggered by hydroxyurea (HU), an inhibitor of ribonucleotide reductase (Rhind and Russel, 1998; Weinert, 1998). In the fission yeast *Schizosaccharomyces pombe*, a number of genes have been identified that are involved in the G2-M checkpoint. One such group includes the genes that are essential for growth and required for DNA replication, such as *cdc18*$^+$, *cut5*$^+$/*rad4*$^+$, *cdt1*$^+$, and *pol1*$^+$. Null mutants of these genes are defective in DNA replication but allow mitotic events inhibiting premature initiation of mitosis (Kelly *et al.*, 1993; Saka and Yanagida, 1993; Saka *et al.*, 1994; Hofmann and Beach, 1994; D'Urso *et al.*, 1995; McFarlane *et al.*, 1997). Involvement of Cdc18, Cdt1, and Pol1 is probably indirect, because replication complexes have to be assembled to establish a checkpoint control over mitosis. On the other hand,

‡ Corresponding author. E-mail address: hnojima@biken.osaka-u.ac.jp.

Table 1. *S. pombe* strains used in this study

Name	Genotype	Source
C525	*h*^{90}/*h*^{90} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	C. Shimoda
KTM103	*h*^{90} *ade6-M216 leu1-32 ura4-D18 rfc3::ura4*$^+$ [pREPS81/*rfc3*$^+$]	This work
MKY2-1	*h*$^+$ *leu1-32 ura4-D18 his7 lys1*	M. Yanagida
MSY101	*h*$^+$ *leu1-32 ura4-D18 his7 rfc3::ura4*$^+$ [pREPS81/*rfc3*$^+$]	This work
MSY10	*h*$^+$ *leu1-32 ura4-D18 his7 rfc3::ura4*$^+$ [pEA500/*rfc3*$^+$]	This work
MSY11	*h*$^+$ *leu1-32 ura4-D18 his7 rfc3::ura4*$^+$ [pEA500/*rfc3-1*]	This work
MSY1	*h*$^+$ *leu1-32 ura4-D18 his7 rfc3-1*	This work
MSY102	*h*$^+$ *ade6-M216 leu1-32 chk1:ep*	N.C. Walworth
MSY22D	*h*$^+$ *ade6-M216 leu1-32 ura4-D18 his7 rfc3-1 chkl:ep*	This work
NP28-7D	*h*$^-$ *leu1-32 ura4-D18*	K. Nabeshima
*rad3*Δ	*h*$^-$ *ade6-704 leu1-32 ura4-D18 rad3::ura4*$^+$	A.M. Carr

Cut5/Rad4 protein seems to play a direct role in the checkpoint control (McFarlane *et al*., 1997).

Another group includes *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, *hus1*$^+$, *chk1*$^+$, *cds1*$^+$, and *crb2*$^+$/*rhp9*$^+$, which are involved in the surveillance of DNA damage and/or incomplete DNA replication (Al-Khodairy and Carr, 1992; Al-Khodairy *et al*., 1994; Enoch *et al*., 1992; Rowley *et al*., 1992; Murakami and Okayama, 1995; Carr, 1997; Saka *et al*., 1997; Willson *et al*., 1997). Because mutations in any one of the first six of these genes, which are known as the checkpoint rad genes, result in very similar phenotypes abolishing both the DNA damage and DNA replication checkpoints, it has been proposed that their gene products form a complex to link both of these checkpoints to G2-M arrest by activation of two downstream kinases, Chk1 and Cds1, by a phosphorylation event (Carr, 1997). It has been shown that an allele of *rad1*$^+$ (Kanter-Smoler *et al*., 1995) and an allele of *rad26*$^+$ (Uchiyama *et al*., 1997) abolished the DNA replication checkpoint alone. Moreover, a mutation in *chk1*$^+$ disrupts the DNA damage checkpoint, and Chk1 is phosphorylated in response to DNA damage but not S-phase arrest (Walworth and Bernards, 1996). Cds1, on the other hand, is phosphorylated and activated by both S-phase arrest and DNA damage, but activation by DNA damage is only seen in S phase, suggesting that Cds1 specifically responds to aberrant replication structures (Lindsay *et al*., 1998). These results suggest that the DNA damage and DNA replication checkpoints function through distinct mechanisms.

When DNA is damaged during G1, S, or G2 phase in the budding yeast *Saccharomyces cerevisiae*, the G1/S checkpoint inhibits the G1-S transition (Siede *et al*., 1993; Siede *et al*., 1994), the intra-S checkpoint decelerates the S phase progression (Paulovich and Hartwell, 1995), and the G2/M checkpoint arrests cells at the G2-M transition (Weinert and Hartwell, 1988). In addition, the meiotic prophase checkpoint delays the meiotic process before meiosis I (Lydall *et al*., 1996), and DNA damage causes transcriptional induction of repair genes (Hwang *et al*., 1998). Another important surveillance mechanism is the DNA replication (S phase) checkpoint, which prevents entry into mitosis when DNA replication is blocked by HU. A number of genes have been shown to be involved in these checkpoint mechanisms. Among these are the nonessential genes *RAD9*, *RAD17*, *RAD24*, *DDC1*, and *MEC3*, which are required for DNA damage checkpoints but not for the DNA replication checkpoint, whereas the essential genes *MEC1* and *RAD53* are required not only for DNA damage checkpoints but also for the DNA replication checkpoint (for review, see Weinert, 1998). These checkpoint factors showed structural and functional similarities between fission yeast and budding yeast. Moreover, recent identification of human homologues of these checkpoint factors further provided evidence that most of the damage response pathways are highly conserved among eukaryotes. Components of the DNA replication complex of budding yeast such as the large subunit of replication protein A, the catalytic subunit of DNA primase, DNA polymerase ε (Pol ε), the *DPB11* gene product, and two subunits of replication factor C (Rfc5 and Rfc2) are also involved in DNA damage checkpoints and/or the DNA replication checkpoint (Araki *et al*., 1995; Navas *et al*., 1995; Sugimoto *et al*., 1996, 1997; Noskov *et al*., 1998).

Budding yeast RFC is a heteropentamer consisting of one large and four small subunits that are encoded by the *RFC1* and *RFC2–5* genes, respectively (Cullmann *et al*., 1995). RFC acts as a primer recognition factor for polymerases δ and ε at the primer–template junction, and the associated ATPase activity is stimulated by the binding of RFC to DNA and then helps to load proliferating cell nuclear antigen (PCNA) onto the DNA template (Tsurimoto and Stillman, 1989; Tsurimoto *et al*., 1990). The amino acid sequences of the RFC subunits are similar to each other and to the *RAD24* gene product, and functional and physical interactions between the subunits of RFC and Rad24 in the checkpoint responses have been demonstrated by genetic and biochemical studies (Shimomura *et al*., 1998). In fission yeast, however, little is known about conservation of this mechanism in the checkpoint pathway. Here we report the isolation of the *rfc3*$^+$ gene of fission yeast and show that it is required for the checkpoint regulation caused by replication blockage and DNA damage. We also demonstrate that Rad17, the fission yeast homologue of the budding yeast *RAD24* gene product, interacts with Rfc3 in vivo.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Media

S. pombe strains used in this study are listed in Table 1. Standard *S. pombe* genetic procedures were followed (Alfa *et al*., 1993). *S. pombe* was grown in standard rich media (YPD or YEL) and in synthetic minimal media (EMM2). For the induction of mating and meiosis, cells were cultured in SPA medium at 25°C (Alfa *et al*., 1993). When specified, adenine, leucine, uracil, histidine, and lysine were sup-

plemented into the media at a concentration of 75 μg/ml. To select diploid cells, Phloxine B (5 μg/ml) was added to YPD medium. The medium was supplemented with 5 μM thiamine for induction of expression using the *nmt* promoter where indicated.

Gene Disruption and Southern Blot Analysis

Using the *rfc3*$^+$ cDNA fragment of *S. pombe* as a probe, we cloned the genomic region encompassing the *rfc3*$^+$ gene from an *S. pombe* genomic library, which was constructed using partial *Sau*3AI DNA fragments inserted into the *Bam*HI site of the Bluescript KS(+) vector (Stratagene, La Jolla, CA). For disruption of the *rfc3*$^+$ gene, a 1.8-kb fragment carrying the *S. pombe ura4*$^+$ gene (Grimm *et al.*, 1988) was inserted between the *Bgl*II and *Bcl*I sites of *rfc3*$^+$ genomic DNA, which resided between exons 4 and 5. The resulting disrupted genes were introduced into the diploid strain C525 (Maekawa *et al.*, 1994), and the Ura$^+$ transformants were screened for disruption of one of the copies of these genes by standard Southern blot analysis (Sambrook *et al.*, 1989). Tetrads from these strains were then dissected.

Sequencing Analysis and Homology Search

Standard manipulations of nucleic acids were performed according to established procedures (Sambrook *et al.*, 1989). Nucleotide sequences were determined by the dideoxy chain termination method using a SequiTherm LC Long-Read cycle sequencing kit (Li-Cor, Lincoln, NE). Sequence homologies were determined using the BLAST and BLASTN algorithms.

Epitope Tagging

To attach the FLAG epitope tag to Rfc3, we synthesized the oligonucleotides FR3N (5′-ATAGGCGCGCCGTCGACAATGTCTATCGAAAAAGGTAAAG-3′) and FR3C (5′-TATTCTTAGCGGCCGCTTATTTACCTTTGCTGCTAAATC-3′), which encompass the open reading frame of Rfc3 and correspond to nucleotides 2271–2292 and 3743–3763 (DDBJ accession number AB017039), respectively. The *Asc*I and *Not*I sites introduced into the oligonucleotides are underlined. Using these oligonucleotides as primers and *rfc3*$^+$ cDNA as a template, we performed a PCR and generated a DNA fragment harboring the open reading frame of Rfc3. This DNA fragment was digested with *Asc*I and *Not*I and inserted into the pGEX-4T-2 vector (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). To select a clone free from point mutations caused by PCR amplification, we randomly picked at least four clones and determined the DNA sequences of the amplified regions. The selected plasmid DNA was digested with *Sal*I and *Not*I and inserted into pRFC1 (Nabeshima, unpublished data), which was designed to fuse cDNA inserts and a FLAG tag in-frame via *Sal*I–*Not*I sites. The plasmid DNA carrying the correct Rfc3-FLAG sequence was then transformed into NP28–7D cells, and the transformants were streaked on EMM2 plates in the presence of 5 μM thiamine at 30°C.

Immunoprecipitation and Western Blotting

NP28–7D cells were transformed with pREP41 or pREP42 vectors carrying either hemagglutinin (HA)-tagged *rad17*$^+$ (pREP41-Rad17H) or FLAG-tagged *rfc3*$^+$ (pREP42-Rfc3F). The transformants were grown in synthetic medium (EMM2) to a density of 5.0×10^6 cells/ml in the absence of thiamine for 18 h. Approximately 1.0×10^9 cells were collected by centrifugation, washed, and resuspended in 200 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na-ortho-vanadate, 15 mM *p*-nitrophenylphosphate, 1 mM dithiothreitol, 0.1% Triton X-100, 40 mM β-glycerophosphate, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 3 μg/ml pepstatin, and 100 μg/ml PMSF). Four hundred microliters of glass beads were added, and the cells were lysed by vortexing. Cell debris was cleared by centrifugation for 30 min at 4°C, and the protein concentration of the extract was determined using a commercial protein assay (Bio-Rad, Hercules, CA). One milligram of the extract was immunoprecipitated with either anti-HA (3F10; Boehringer Mannheim, Mannheim, Germany) or anti-FLAG (M2 for FLAG; Eastman Kodak, New Haven, CT) monoclonal antibodies by overnight incubation at 4°C and then incubated for 2 h with 30 μl of protein G-Sepharose beads. Immunoprecipitates were washed and boiled in SDS-PAGE sample buffer as described previously (Sugimoto *et al.*, 1997). For Western blot analysis, the proteins were separated by SDS-PAGE (10% polyacrylamide) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking treatment, anti-HA antibodies and anti-FLAG antibodies were used to probe the blots, and the bands recognized by antibodies were visualized using the Renaissance system (DuPont New England Nuclear, Boston, MA).

Random Mutagenesis and Isolation of Temperature-sensitive Mutants

To introduce a point mutation into the *rfc3*$^+$ gene at random, PCR (one cycle of 95°C for 2 min and then 30 cycles of 95°C for 45 s, 56°C for 45 s, and 72°C for 2 min with *Taq* polymerase) was conducted with the plasmid DNA carrying the *rfc3*$^+$ gene as a template and the following oligonucleotides as primers: oligonucleotide N1 upstream of the ATG codon, 5′-GCAGCTTACTAGTGTGTCCACCGTACATTGTATG-3′ (corresponding to nucleotides 2050–2070; DNA Data Bank of Japan (DDBJ) accession number AB017039); and N2 for the region surrounding the termination codon, 5′-CGTACTCGAGGAGTTTTTAACAATTTATATTG-3′ (corresponding to nucleotides 3883–3904). The *Spe*I and *Xho*I restriction sites introduced into the oligonucleotides are underlined. The 1.9-kb amplicon was digested with *Spe*I and *Xho*I, ligated into the *Spe*I–*Xho*I sites of pEA500 (Apolinario *et al.*, 1993), and propagated in *Escherichia coli*. This plasmid DNA pool of mutagenized *rfc3* genes was transformed into an *rfc3*-null strain, MSY101, carrying the wild-type *rfc3*$^+$ gene in pREPS81, a novel version of pREP81 containing the *LEU2* gene as a marker (Tanaka, unpublished data). After 6 d of incubation at 25°C, ~1600 Leu$^+$ His$^+$ colonies were streaked onto EMM2 plus leucine to remove the pREPS81-*rfc3*$^+$ cDNA. We found 1100 Leu$^-$ His$^+$ colonies that contained the mutagenized *rfc3* genes alone. We then tested growth profiles by replica plating onto EMM2 plus leucine plates and subsequent incubation at either 28 or 37°C. Finally, we obtained four candidate strains of temperature-sensitive mutants. Plasmid DNA was recovered from these strains, and the mutated sites were determined by DNA sequencing. Cell morphology was monitored with a microscope (Axiophot; Zeiss, Oberkochen, Germany) and recorded with a charge-coupled device camera (Photometrics PXL1400; Roper Scientific, Trenton, NJ).

We integrated the *rfc3-1* genomic sequence into its genomic locus as follows. The *Pst*I–*Sal*I genomic fragment of *rfc3-1* was introduced into the *rfc3::ura4*$^+$ deletion strain carrying the plasmid pEA500-*rfc3-1* (MSY11), and the transformants were selected by resistance to 5-fluoroorotic acid (Grimm *et al.*, 1988). Genomic integration and the correct replacement of *ura4*$^+$ marker in the *rfc3*Δ strain by the mutated *rfc3-1* gene were confirmed by Southern blot analysis and DNA sequencing.

Pulsed Field Gel Electrophoresis (PFGE)

The procedures for PFGE were described previously (Kelly *et al.*, 1993). Wild-type (MSY10) and *rfc3-1* cells (MSY11) were grown at 28°C and then shifted up to 37°C for 23 h. Cells were collected and treated for PFGE. PFGE was conducted in 0.6% chromosomal grade agarose (Bio-Rad) in a Bio-Rad CHEF-Mapper system at 14°C for 72 h at 50 V in 0.5× TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA), with a switch time of 30 min.

Flow Cytometry

Cells were fixed in ice-cold 70% ethanol and stained for cytometry with propidium iodide according to the standard protocol (Alfa *et*

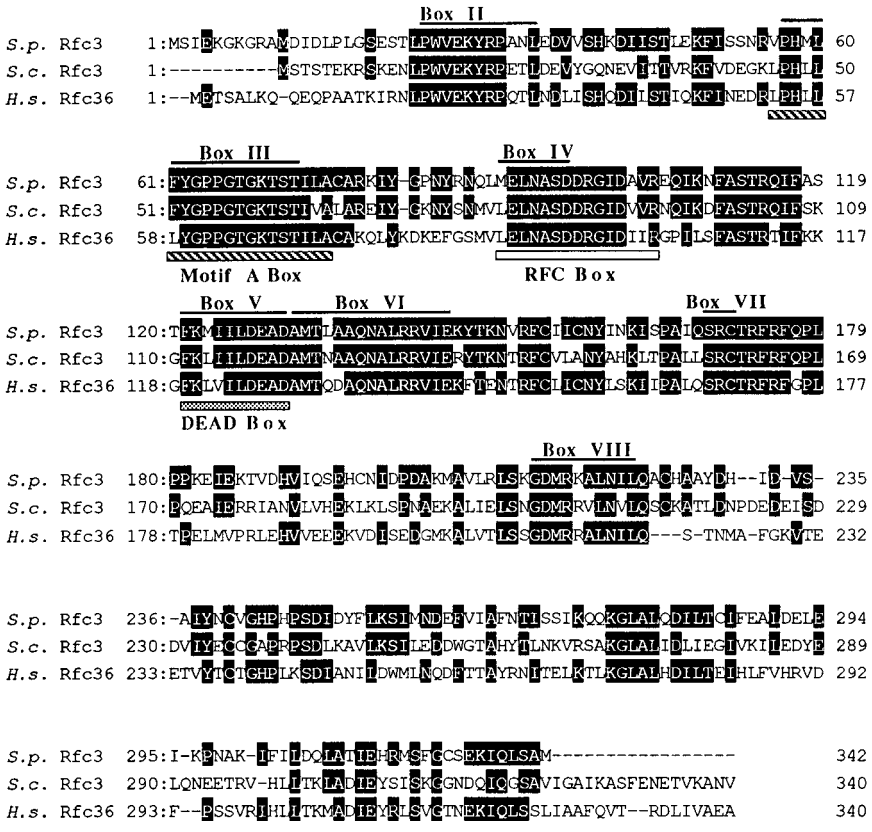

Figure 1. Fission yeast Rfc3 is homologous to budding yeast and human Rfc3. Identical amino acids are indicated by darkly shaded patterns. Spaces (indicated by hyphens) have been inserted to optimize alignment. Locations of motifs conserved in all RFC subunits such as RFC boxes II–VIII, motif A box, RFC box, and DEAD box are indicated. The accession numbers of the *rfc3*$^+$ cDNA and the genomic DNA sequence surrounding the *rfc3*$^+$ gene in the DDBJ–European Molecular Biology Laboratory–GenBank database are AB017040 and AB017039, respectively.

al., 1993). Flow cytometry was performed on a FACScan cell sorter, and data analysis was carried out using CELL QUEST software for the Macintosh computer (Becton Dickinson, Nutley, NJ).

Survival Analysis

To examine a sensitivity to HU or MMS, 10 mM HU or 0.007% MMS was added to yeast cells, which were grown at 28°C to early log phase. Aliquots of the cells were removed at appropriate intervals, diluted, and plated. After incubation at 28°C for 4 d, colonies were counted, and the survival rates were expressed as a percentage relative to control samples, which were plated immediately before addition of HU or MMS. For gamma ray sensitivity analysis, freshly grown cells were irradiated from a ^{60}Co source at a dose rate of 234 Gy/h. For UV sensitivity analysis, freshly grown cells were spread on plates and exposed to the appropriate dose of UV irradiation using the dosage settings of a Stratagene Stratalinker 2400. After irradiation, appropriately diluted samples were plated, which were then incubated at 28°C for 4 d to allow colony growth and counting.

Preparation and Analysis of Synchronous Cultures

An elutriation procedure to prepare a synchronous culture was performed according to the method of Edwards and Carr (1997). Samples were divided and subjected to appropriate treatments with HU, MMS, and gamma and UV irradiation. Aliquots were extracted at 15- or 30-min intervals and fixed in methanol. The proportion of mitotic or septated cells was scored by estimation of the cell cycle position by DAPI and calcofluor staining.

Chk1 Phosphorylation

Cells were grown to midlog phase and treated with 0.1% MMS, 20 Gy of gamma irradiation, or 45 J/m^2 of UV irradiation at a permissive temperature. After incubation for 1 h, lysates were prepared and analyzed by mobility shift after immunoblotting with anti-HA antibody (12CA5; Boehringer Mannheim).

RESULTS

Isolation and Structure of rfc3$^+$ *Gene*

During the course of a large-scale isolation of meiosis-specific genes of *S. pombe* using a cDNA subtraction strategy, we identified a cDNA clone (*rfc3*$^+$) encoding a protein similar to the budding yeast RFC3 as one of the genes whose transcription was moderately induced during meiosis (our unpublished results). The *rfc3*$^+$ cDNA encoded a neutral protein (pKa = 7.8) of 342 amino acids with a calculated molecular weight of 38,439. Using the *rfc3*$^+$ cDNA as a probe, we screened a genomic library of *S. pombe* (*h*$^-$ L972), cloned a DNA fragment including the surrounding regions of the *rfc3*$^+$ gene, and determined the DNA sequence. Comparison of the genomic DNA and cDNA sequences indicated that the *rfc3*$^+$ gene was split by five introns, which conformed to known consensus sequence motifs at the intron–exon junctions and contained sequences matching the consensus for an internal branch point. A gene consisting of six exons is unusual in *S. pombe*.

Figure 1 shows the alignment of Rfc3 proteins from fission yeast, budding yeast (Cullmann *et al.*, 1995), and human (Tsurimoto and Stillman, 1989). Their sizes are similar, and the identical amino acids (shown by light shading) are apparently concentrated in the N-terminal half of the molecule.

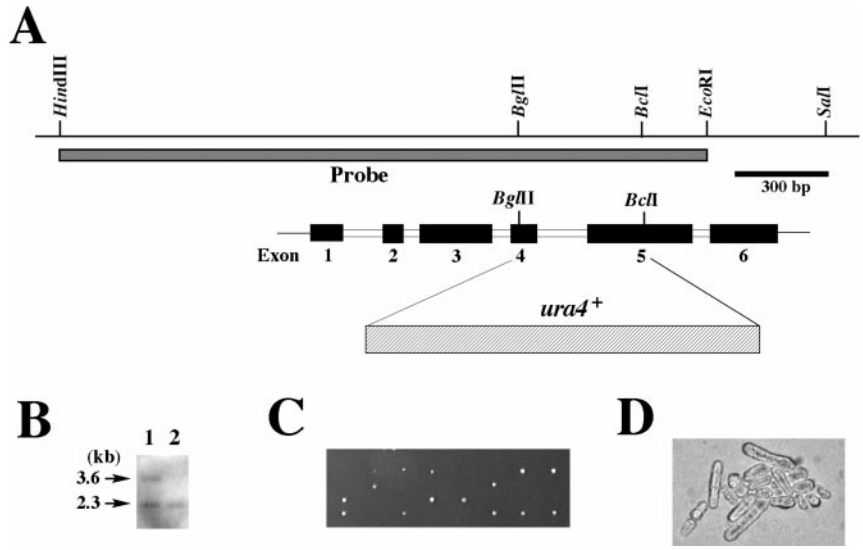

Figure 2. Disruption of *rfc3*$^+$ caused a lethal phenotype. (A) Restriction map of the *rfc3*$^+$ gene and the surrounding regions. The *rfc3*$^+$ gene consists of six exons, which are shown by filled boxes. To disrupt the *rfc3*$^+$ gene, the *ura4*$^+$ gene was inserted between exons 4 and 5. The region deleted by replacement with the *ura4*$^+$ gene is depicted as a shaded box. (B) Detection of the disrupted chromosomal *rfc3*$^+$ gene of the diploid strain carrying *rfc3::ura4*$^+$ on one chromosome by Southern blot analysis with the *Hin*dIII–*Eco*RI fragment as a probe (shown as a gray box in A). Genomic DNA was prepared from each strain, digested with *Eco*RI and *Hin*dIII, separated by 1% agarose gel electrophoresis, and blotted onto a nylon membrane. Among a set of haploid progenies derived from this diploid strain, two (lanes 1 and 2) were judged to contain *ura4*$^+$ inserts. (C) Photograph of tetrads dissected from a diploid heterozygous for *rfc3* replacement to show that the *rfc3*$^+$ gene is essential to vegetative growth. (D) Typical example of the terminal morphology of the germinated *rfc3::ura4*$^+$ spores. Most deduced *rfc3*$^-$ spores completed germination, divided four to five times, and ceased growth, presumably after consuming the residual *rfc3*$^+$ activity.

Sequences known to be conserved among Rfc proteins (Cullmann *et al.*, 1995) such as RFC box II, -III, -IV, -V, -VI, -VII, and -VIII are highly conserved among these Rfc3 proteins. Other notable conserved sequences are the motif A box and DEAD box, which are also characteristic of Rfc proteins. Thus, we conclude that the gene we isolated here encodes a fission yeast counterpart of Rfc3.

Rfc3 Is Essential to Growth

To examine whether the *rfc3*$^+$ gene was essential for yeast cell growth, we constructed an *rfc3*-disrupted mutant by one-step gene replacement. As illustrated in Figure 2A, the *Bgl*II–*Bcl*I fragment that was located between exons 4 and 5 was replaced with a 1.8-kb DNA fragment carrying the *S. pombe ura4*$^+$ gene. Diploid cells with one *rfc3*$^+$ gene replaced by *ura4*$^+$ sporulated and germinated with a segregation ratio of 1:1 to the wild type. We confirmed the successful disruption of the *rfc3*$^+$ gene by testing for the presence or absence of the uracil auxotrophic marker and by Southern blot analysis (Figure 2B). As shown in Figure 2C, the spores that resulted from the cell having a disrupted *rfc3*$^+$ gene did not form colonies, indicating that *rfc3*$^+$ is essential for cell growth. The nonviable spores gave rise to microcolonies with slightly unusual morphology (Figure 2D). Most deduced *rfc3*$^-$ spores completed germination, divided four to five times, and ceased growth, presumably after consuming the residual Rfc3 activity carried over from mother cells.

Isolation of a Temperature-sensitive Mutant, rfc3-1

To characterize the essential domains and activities of the third subunit of RFC, we used a genetic approach to isolate and characterize *rfc3* mutants generated by random PCR mutagenesis. A mutagenized *rfc3* gene library was used to transform MSY101, in which the *rfc3*$^+$ gene is deleted from the chromosomes but which carries multicopy plasmids containing the *rfc3*$^+$ cDNA. After plasmid shuffling, ~1100 transformants carrying the mutagenized *rfc3* gene were examined for temperature sensitivity. We isolated four temperature-sensitive mutants, all of which grew normally at 28°C but poorly at 37°C when compared by replica plating. One of them, *rfc3-1*, was selected for further analysis.

To determine the site of the mutation in *rfc3-1*, the mutated gene was amplified from *rfc3-1* alleles by PCR and sequenced. As a result, the mutation in *rfc3-1* was found to consist of a single nucleotide change (from A to T) at base 1052, which resulted in a change from R to W at amino acid position 216 (Figure 3, B and C). This region of the *rfc3* gene has extensive similarity with Rfc3 of *S. cerevisiae* and the 36-kDa subunit of human RFC, suggesting that this region is important for the specific function of these genes.

Phenotype of the rfc3-1 Mutant

RFC is known to load PCNA onto the primer terminus, after which the polymerase binds to this DNA–RFC–PCNA complex to form a processive replication complex. In addition to the role of the RFC complex in chromosomal DNA replication, the *RFC2* and *RFC5* genes of *S. cerevisiae* have been shown to play a role in replication checkpoint function (Sugimoto *et al.*, 1996; Noskov *et al.*, 1998). Replication checkpoint deficiencies would be expected to cause rapid loss of cell viability, because the cell would enter mitosis with incompletely replicated DNA.

We first wished to examine whether DNA replication was also defective in the *rfc3-1* mutant. For this purpose, we monitored the number, viability, DNA content, and morphology of exponentially growing *rfc3-1* cells (MSY11) at either 28 or 37°C (Figure 4A). The *rfc3-1* cells gradually lost viability during the time course of incubation at the restrictive temperature (37°C). After 24 h at 37°C, ~90% of the *rfc3-1* cells became nonviable. To investigate the point in the cell cycle at which the *rfc3-1* cells arrested, DNA content was determined by fluorescence-activated cell sorter analysis af-

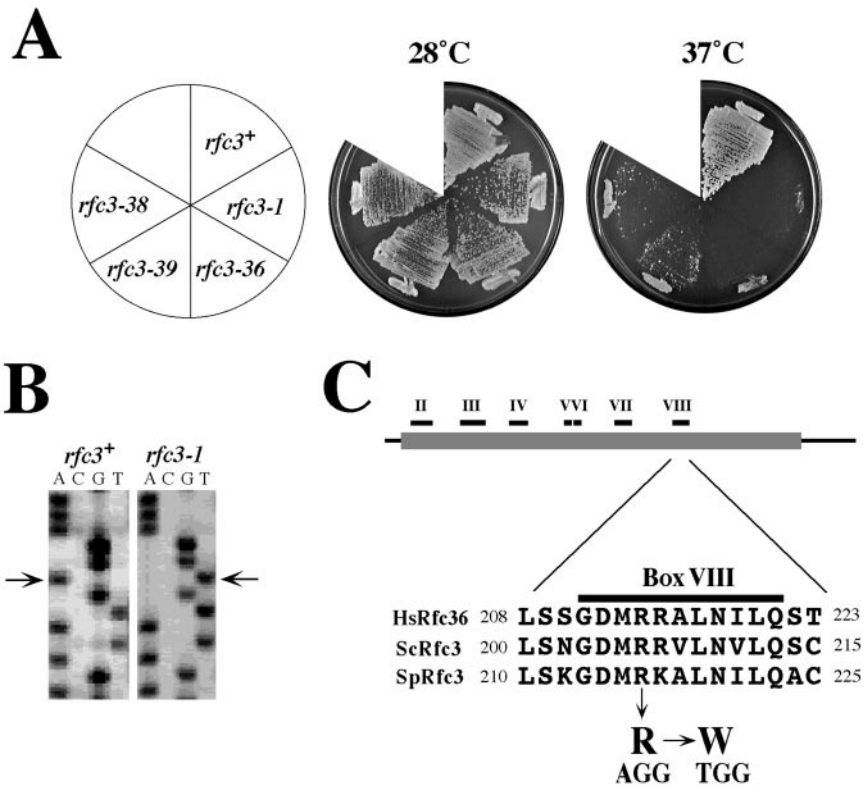

Figure 3. Isolation and characterization of the *rfc3-1* mutant. (A) Four of the isolated *rfc3* mutant cells (*rfc3-1, -36, -38,* and *-39*) showed poor growth at 37°C compared with the wild type (*rfc3*$^+$). Among them, *rfc3-1* and *-36* showed more severe temperature sensitivities. (B) Sequencing ladder in the vicinity of the mutation site in *rfc3-1* showing a point mutation (A to T). (C) A point mutation found in the box VIII domain of the *rfc3*$^+$ gene converted the arginine at position 216 to tryptophan. The amino acid sequences in the vicinity of the mutation site are highly conserved among Rfc3 proteins of budding yeast and the human 36-kDa RFC subunit.

ter a temperature shift to 37°C, and the results were compared with those of *rfc3*$^+$ cells (MSY10; Figure 4B). Although the *rfc3*$^+$ cells had a major peak at 2C DNA, *rfc3-1* mutant cells displayed a broad peak. This indicates that *rfc3-1* cells, after incubation at 37°C, were unable to divide evenly and generated cell populations with fragmented chromosomes. Cell morphology and distribution of nuclei as visualized by DAPI staining were observed under a microscope. As shown in Figure 4C, cells with fragmented nuclear DNA, which was generated by unequal segregation of DNA between mother and daughter cells, were frequently observed. The proportion of such abnormal nuclei increased during the time course of incubation at 37°C.

PFGE of rfc3-1 DNA

PFGE was used to examine whether the cells were arrested in S phase and to determine the stage of replication reached by chromosomal DNA in *rfc3-1* mutant cells at 37°C. To provide quantitative results, we performed Southern blot analysis using the *aro3*$^+$ gene, which is located in chromosome I. As shown in Figure 5, when a large amount of chromosomal DNA from *rfc3*$^+$ cells (MSY10) entered the gel, only a small portion of DNA prepared from *rfc3-1* cells (MSY11) entered the gel during the time course of incubation at the restrictive temperature (12 and 23 h). As a control for DNA whose synthesis was blocked during S phase, we used chromosomal DNA of wild-type cells treated with a DNA synthesis inhibitor, HU. As shown in Figure 5B, leftmost lane, the control DNA failed to enter the gel in a manner similar to that observed in the DNA extracted from *rfc3-1* cells. These results suggest that *rfc3-1* cells cannot start DNA replication properly at the restrictive temperature, indicating that Rfc3 plays a pivotal role in DNA replication.

rfc3-1 Mutant Displays a Replication Checkpoint Defect

We next examined the effect of HU on the phenotype of *rfc3-1*. We added HU to a final concentration of 10 mM to exponentially growing wild-type (*h*$^-$ L972), *rfc3-1* (MSY1), and *rad3*Δ cells at 28°C. As shown in Figure 6A, *rfc3-1* was extremely sensitive to treatment with HU even at the permissive temperature (28°C): <1% of the cells survived a 12-h incubation in 10 mM HU. The rate of decrease in viability of *rfc3-1* cells as incubation proceeded was less dramatic than that of *rad3*Δ cells, which were examined in parallel as a control. Under these conditions, the cell cycle was blocked in *rfc3*$^+$ cells because of the checkpoint control mechanism, but the cells continued to synthesize RNA and protein, resulting in elongated, unseptated cells with a single nucleus (Figure 6A, right panel). In contrast, a strikingly different phenotype was observed when *rfc3-1* cells were incubated in the presence of 10 mM HU. Typical examples of cells with abnormal morphology after 12 h of incubation are shown in Figure 6A (right panel, white arrows). To test whether this sensitivity was caused by a replication checkpoint defect, we have measured the duration of the checkpoint delay at the permissive temperature (26°C) (Figure 7A). Compared with wild-type (*h*$^-$ L972) cells, *rfc3-1* (MSY1) cells did not arrest the cell cycle, although the entry into M phase was delayed

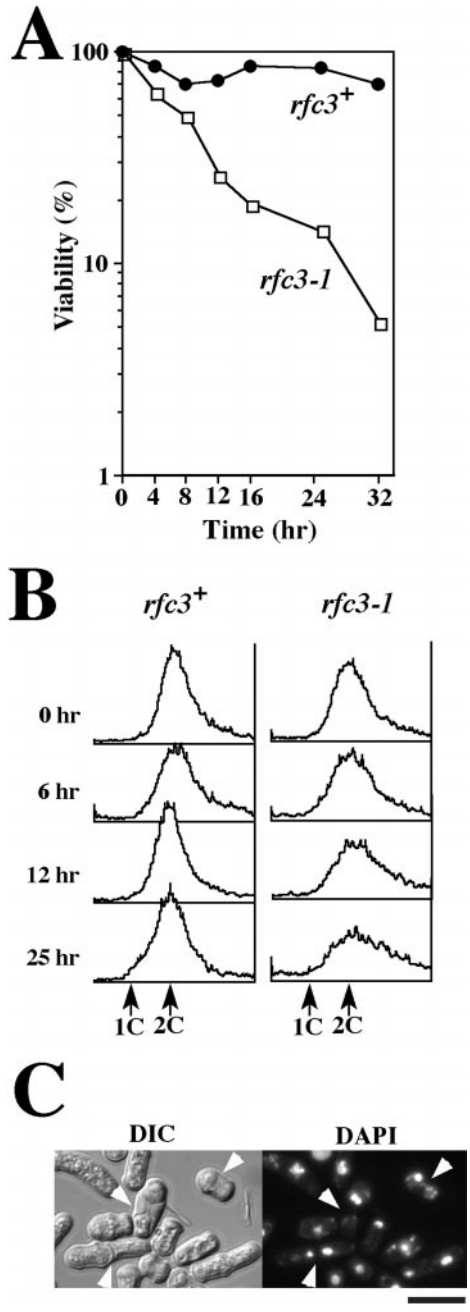

Figure 4. Growth phenotypes of the *rfc3-1* mutant. (A) Cell viability of the *rfc3*$^+$ (MSY10) and the *rfc3-1* mutant (MSY11). Exponentially growing cells at 25°C were shifted to incubation at 37°C (time = 0). After 4, 8, 12, 16, 25, and 32 h, a fixed number of cells were removed, diluted, plated onto EMM2 plates supplemented with leucine, and incubated at 28°C for 4 d. Colonies were scored, and viability was expressed as a percentage of the colonies that formed on samples plated immediately before temperature shift. (B) The DNA content of *rfc3*$^+$ and *rfc3-1* cells incubated at 37°C for 0–25 h was estimated by the FACScan method. Positions of the cells with 1C and 2C DNA are shown by arrows. (C) Morphology of cells incubated at 37°C for 32 h. DNA was visualized by staining cells with DAPI. In the *rfc3-1* mutant, cells with abnormal nuclear divisions are denoted by arrowheads. Cell morphology was recorded with a charge-coupled device camera. Bar, 10 μm.

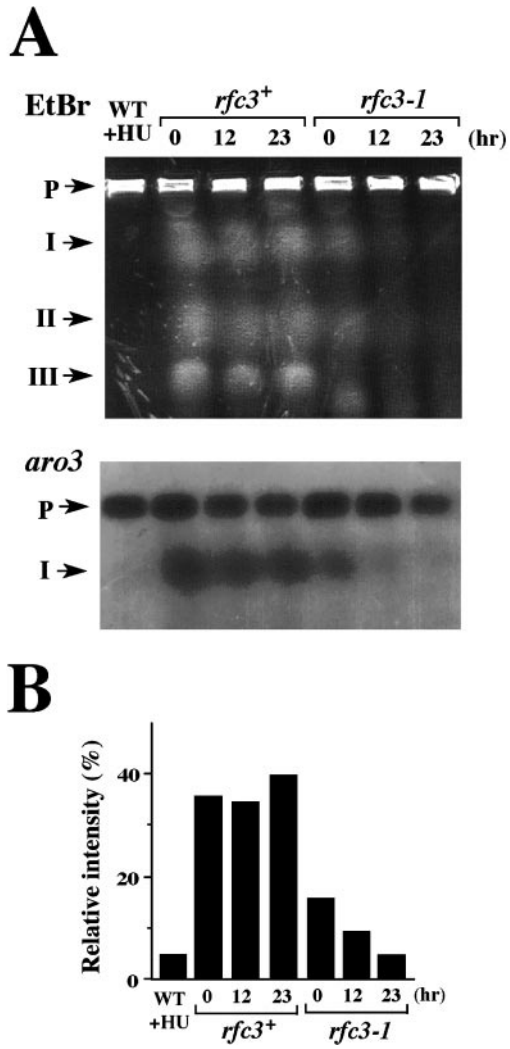

Figure 5. PFGE demonstrates that most of the chromosomes of *rfc3-1* cells at a restrictive temperature were abnormally replicated. (A) PFGE profile of *rfc3*$^+$ (MSY10) and *rfc3-1* (MSY11) cells incubated at 37°C for 12 or 23 h. DNA from wild-type (*h*$^-$ L972) cells exposed to 30 mM HU for 5 h was also examined as a control for abnormally replicated DNA. The agarose gel was analyzed by Southern blotting and probing with a ^{32}P-labeled *aro3*$^+$ gene, which is present in chromosome I. The top row of bands was derived from the wells containing the residual material that was not able to migrate into the gel. P, agarose plug at the origin of electrophoresis; I, position of *S. pombe* chromosome I. (B) Quantitative analysis of the bands indicating that a large proportion of the DNA from *rfc3-1* cells incubated at 37°C for 12 or 23 h and from *rfc3*$^+$ cells exposed to HU did not enter the gel. The relative intensity of band I of each lane as normalized by the intensity of band P is indicated by a filled bar.

significantly (Figure 7A, right panel). This is reminiscent of the situation seen in one of the *rad17* mutants (K118E), which showed a partial defect in replication checkpoint (Griffiths *et al.*, 1995). These results indicate that the replication checkpoint triggered by HU is defective in *rfc3-1* mutant cells, although it is not completely abolished.

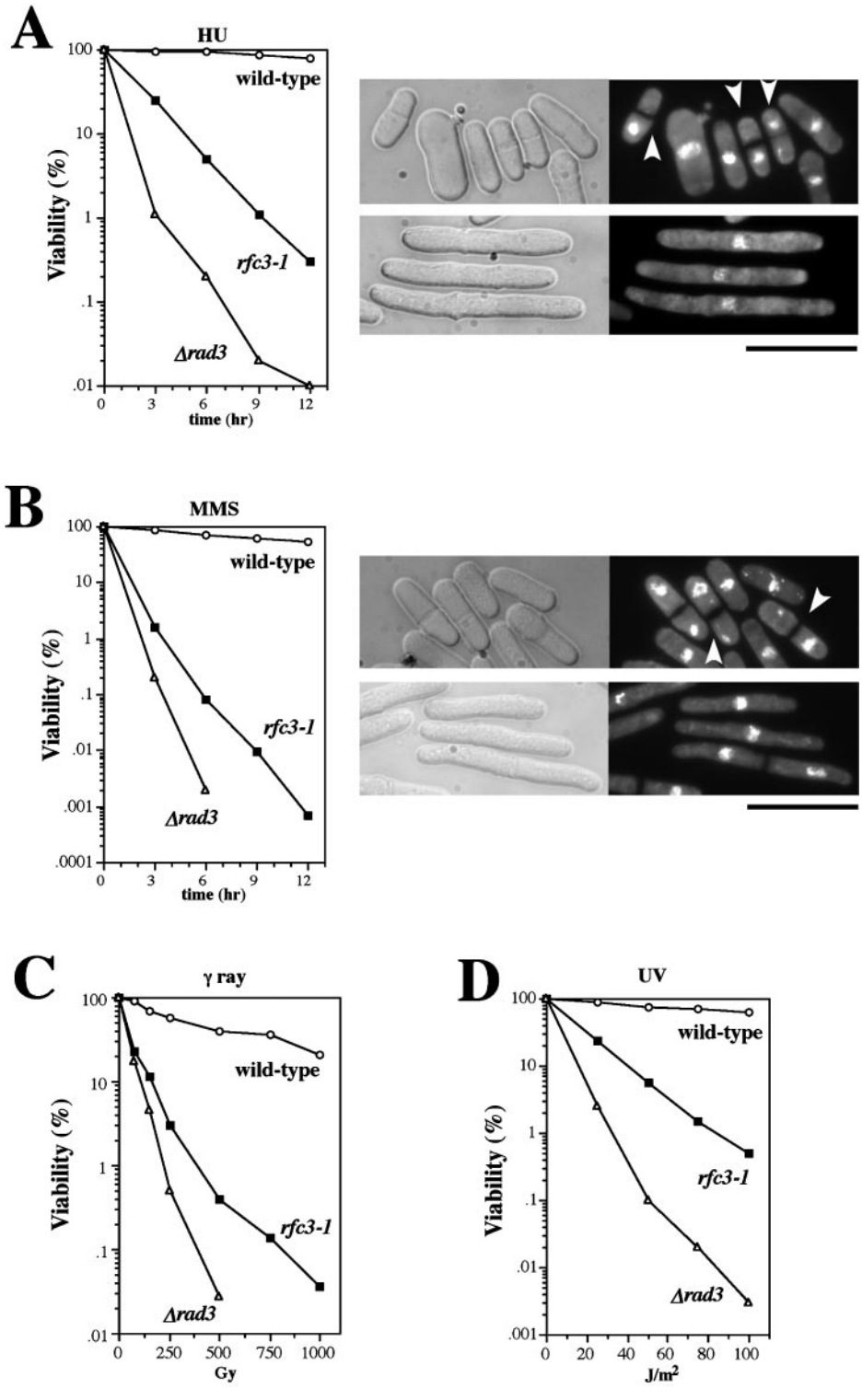

Figure 6. The *rfc3-1* mutant is sensitive to HU, MMS, and gamma and UV irradiation at permissive temperature. Ten micromolar HU (A) or 0.007% MMS (B) was added to the exponentially growing *rfc3-1* (MSY1), wild-type, and *rad3*Δ cells at 28°C (time = 0), and incubation was continued. After 3, 6, 9, and 12 h, cells were collected, diluted, plated, and further incubated at 28°C for 4 d. Colonies were scored, and viability was expressed as a percentage of the colonies that formed on samples plated immediately before addition of HU or MMS. Cell morphology was monitored with a microscope after these treatments for 12 h (A and B, right). In the *rfc3-1* mutant, abnormal nuclear divisions were detected as shown by arrows. Bar, 10 μm. (C) For gamma irradiation, cells were grown to early log phase in the YPD medium, removed, diluted, and irradiated by gamma rays from a ^{60}Co source at a dose rate of 234 Gy/h. After irradiation, appropriately diluted samples were plated and incubated at 28°C for 4 d, when the survival rates were measured by counting the colonies. (D) For UV irradiation, freshly grown cells were spread on YPD plates, and the plates were immediately exposed to the appropriate dose of UV irradiation. The survival rates were measured by counting the colonies after a 4-d culture at 28°C.

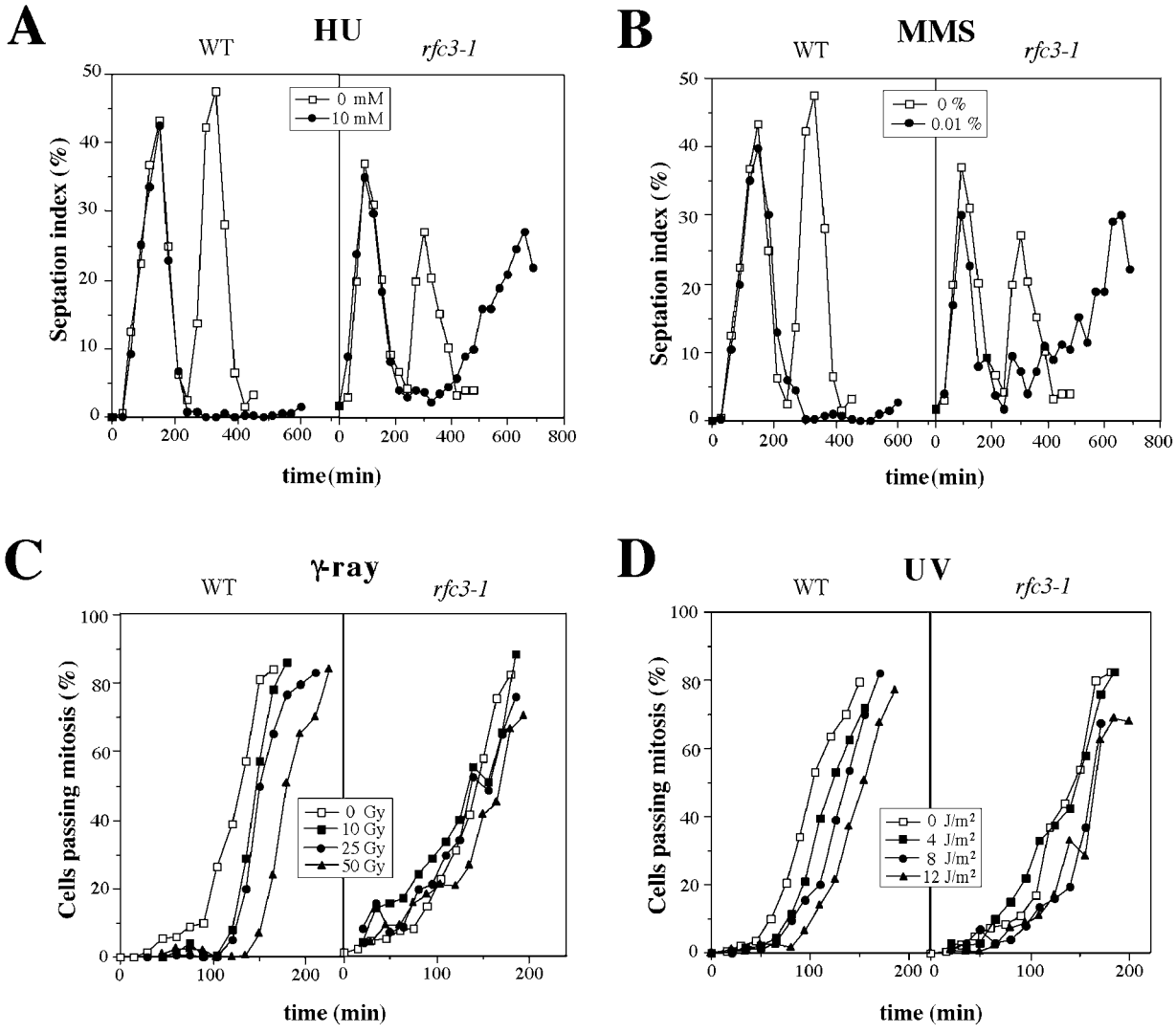

Figure 7. Analysis of the replication and damage checkpoint of *rfc3-1* cells at permissive temperature (26°C). Synchronous cultures of wild-type (h^- L972) and *rfc3-1* (MSY1) cells were generated by elutriation and incubated in the presence of 10 mM HU (A) and 0.01% MMS (B) or exposed to gamma (C) and UV (D) irradiation. The percentages of septated cells or cells passing mitosis were estimated by DAPI and calcofluor staining.

rfc3-1 Mutant Displays a Damage Checkpoint Defect

DNA replication and DNA damage checkpoints are known to share overlapping regulatory components in both budding and fission yeast. We therefore examined whether *rfc3-1* mutant cells were responsive to DNA-damaging agents such as MMS and gamma or UV irradiation. The responses of the *rfc3-1* mutants (MSY1) were compared with those of wild-type (h^- L972) and *rad3*Δ cells, which are known to be defective in the DNA damage checkpoint. As shown in Figure 6, B, left panel, C, and D, under conditions that allowed a large proportion of wild-type cells to remain viable, the *rfc3-1* mutant cells were significantly sensitive to these DNA-damaging agents even at the permissive temperature (28°C). Under these conditions, the *rad3*Δ mutant used as a control was extremely sensitive to these agents, in accordance with previous reports (Al-Khodairy and Carr, 1992).

The sensitivities of the *rfc3-1* mutant cells to the DNA-damaging agents were found to be directly proportional to the appearance of a cell population with abnormal nuclear morphology, when DAPI-stained cells were observed under a microscope (Figure 6B, right panel). Many of the daughter cells showed unevenly distributed chromosomes after cell division, and some of them displayed an anucleate phenotype (Figure 6B, right panel, arrowheads). It is of note that some MMS-treated cells showed cut phenotypes, which is indicative of a failure of the replication checkpoint just like HU-treated cells. This is probably because the concentration of MMS here was low (0.007%). We could not use a higher

MMS concentration, however, because most *rfc3-1* cells died. At 0.007% MMS, *rad3* mutant cells also exhibited such cut phenotypes like *rfc3-1* cells (our unpublished results). Thus, at such low MMS concentrations, MMS also caused S phase delay, and some of the results (Figure 6B) may become similar to those of HU (Figure 6A).

To further test whether these sensitivities were caused by damage checkpoint defect, we measured the duration of the checkpoint delay at the permissive temperature (26°C) (Figure 7, B–D). As shown in Figure 7B, the ability of wild-type (h^- L972) and *rfc3-1* (MSY1) cells to arrest mitosis after incubation in 0.01% MMS was significantly different. Under a condition in which cells were arrested, *rfc3-1* cells displayed a delayed mitosis, indicating a partial defect in checkpoint arrest incurred by MMS. The reason why wild-type cells divide once after the addition of MMS is again because the concentration of MMS we used here was low (0.01%). At this MMS concentration in wild-type cells, it may be that only some modification occurred without fully invoking the damage checkpoint. Thus cells entered the first round of M phase. If a certain amount of modifications of DNA is sensed at the next round of S phase, then the S-phase checkpoint caused the cell cycle to arrest. When we used 0.05% MMS, wild-type cells never entered M phase, and no peak was observed during the 720 min of observation (our unpublished results). At this concentration, however, most *rfc3-1* cells die, and we cannot compare the results between wild-type and *rfc3-1* cells.

Gamma irradiation provided more clear-cut results. After exposure to gamma rays, synchronous cultures of *rfc3-1* cells displayed no significant delay in cell cycle progression (Figure 7C), indicating that *rfc3-1* cells lacked cell cycle arrest caused by the damage checkpoint. Intriguingly, only a weak difference was seen between the timing of mitosis after exposure to UV irradiation of a synchronous culture of wild-type (h^- L972) and *rfc3-1* cells (MSY1). These results imply that Rfc3 is required for the DNA damage checkpoint and that the checkpoint response of *rfc3-1* cells to MMS and gamma or UV irradiation is somewhat different.

Association of Rfc3 with Other Checkpoint Factors

The amino acid sequence of Rfc3 has been shown to be similar to that of a known checkpoint protein, Rad17 (Griffiths *et al.*, 1995). Considering that the five subunits of RFC, which share similar structural homology, associate in vivo, we reasoned that Rfc3 and Rad17 may also associate with each other. To examine their biochemical interaction in vivo, we tagged the $rfc3^+$ gene with the FLAG epitope and expressed it together with HA-tagged Rad17 using an attenuated version of the *nmt1* promoter (Griffiths *et al.*, 1995). NP28–7D cells were transformed with combinations of pREP41-Rad17H, pREP42-Rfc3F, and the parent vectors (Figure 8), and extracts were prepared from the transformed cells. These extracts were subjected to immunoprecipitation and immunoblotting analysis with either anti-HA or anti-FLAG antibody. When the cell extract carrying both pREP41-Rad17H and pREP42-Rfc3F plasmids was immunoprecipitated by anti-HA antibody and the immunoblot was probed with anti-FLAG antibody, a band migrating at ~40 kDa, corresponding to the expected size of Rfc3-FLAG protein, was detected (Figure 8, upper panel, lane 1). In contrast, no band was seen in the same position in cells carrying the parent vector as a cotransformation partner (Figure 8, upper panel, lanes 2, 3, 5, and 8).

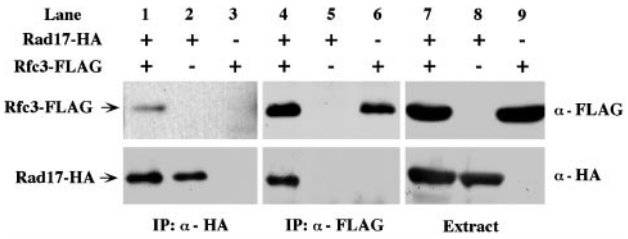

Figure 8. Rfc3 was associated with other checkpoint factors. Association of Rad17 and Rfc3 in vivo. Extracts prepared from NP28–7D cells transformed with plasmids carrying an attenuated *nmt* promoter, expressing both (+/+) or either one of the HA-tagged Rad17 (pREP41-Rad17H) and FLAG-tagged Rfc3 (pREP42-Rfc3F) proteins (+/− or −/+), were immunoprecipitated (IP) with either anti-HA or anti-FLAG antibody. The immunocomplexes were separated by SDS-PAGE, and the duplicate Western blots were probed either with anti-HA (lower panel) or anti-FLAG (upper panel) antibody. The arrow denotes the location of the band representing either HA-tagged Rad17 or FLAG-tagged Rfc3.

Similarly, a band of 80 kDa, corresponding to the expected size of the Rad17-HA protein, was observed in reciprocal immunoprecipitation and immunoblotting experiments in which extracts of cells carrying both pREP41-Rad17H and pREP42-Rfc3F plasmids were immunoprecipitated with anti-FLAG antibody and the immunoblot was probed with anti-HA antibody (Figure 8, lower panel, lane 4). The identity of the Rad17-HA band was confirmed by immunoblotting the anti-HA immunoprecipitate of the extract or the extract itself and subsequent probing of the immunoblots with anti-HA antibody (Figure 8, lower panel, lanes 1, 2, 7, and 8). Similarly, no band was detected in the same position in the cells harboring the parent vector as a cotransformation partner (Figure 8, lower panel, lanes 3, 5, 6, and 9). These results indicate that Rfc3 and Rad17 proteins associate biochemically in vivo.

Rfc3 Functions in a Chk1-dependent Checkpoint Pathway

The protein kinase Chk1 was phosphorylated in response to DNA-damaging agents. Phosphorylation of Chk1 is dependent on several components of the checkpoint pathway, *rad1*, *rad3*, *rad9*, *rad17*, and *rad26* (Walworth and Bernards, 1996). To identify whether Rfc3 functions upstream of Chk1, we assayed the ability of *rfc3-1* mutant to activate Chk1 kinase in response to DNA damage. We constructed a strain carrying both *rfc3-1* mutation and an epitope-tagged allele of *chk1* (Walworth and Bernards, 1996). Asynchronous cultured cells were exposed to 0.1% MMS, 20 Gy of gamma irradiation, or 45 J/m^2 UV irradiation for 1 h at permissive temperature (28°C), and lysates were prepared for Western blot analysis. As shown in Figure 9, the upper band of Chk1 corresponding to the phosphorylated form of Chk1 was almost abolished in the *rfc3-1* mutant. With a longer exposure time, it was observed faintly, probably because the Rfc3-1 protein was partially active at a permissive temperature. This could reflect the fact that the *rfc3-1* mutant results in a partial checkpoint defect caused by HU and DNA-

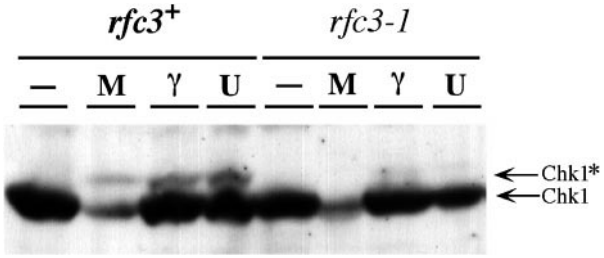

Figure 9. Rfc3 functions in a Chk1-dependent checkpoint pathway. Phosphorylation of Chk1 caused by DNA damage is reduced in *rfc3-1* cells. The phosphorylation-dependent mobility shift of Chk1 kinase was assayed in *rfc3*$^+$ (MSY102) and *rfc3-1* mutant cells (MSY22D) containing the HA-tagged *chk1*$^+$ gene. Cells were grown to midlog phase and treated with 0.1% MMS (M), 20 Gy of gamma irradiation (γ), and 45 J/m^2 UV irradiation (U). Samples without any treatments (−) were also electrophoresed as controls. The phosphorylation status of Chk1 was investigated by SDS-PAGE and Western blot analysis. Chk1*, phosphorylated form of Chk1.

damaging agents (Figure 7). The result suggests that the checkpoint signal emitted from Rfc3 is linked at least to the downstream checkpoint factor Chk1.

DISCUSSION

In the present study, we have isolated the *rfc3*$^+$ gene, which encodes a subunit of the RFC complex of *S. pombe* (Figure 1) and have generated and characterized temperature-sensitive mutations in the *rfc3*$^+$ gene. One of the mutants, *rfc3-1*, was found to harbor a point mutation causing an amino acid replacement in the conserved domain of box VIII (Figure 3). The results presented here demonstrate that Rfc3 of *S. pombe* is essential for DNA replication: disruption of the *rfc3*$^+$ gene was lethal (Figure 2), and the DNA synthesized in the *rfc3-1* cells failed to enter the gel in PFGE (Figure 5). When wild-type cells were exposed to HU, a competitive inhibitor of ribonucleotide reductase, which blocks replication after initiation by preventing nucleotide synthesis, cells arrested at S phase and remained viable without displaying elongated mitotic spindles. In contrast, even at the permissive temperature, the *rfc3-1* mutant cells were sensitive to HU, displaying <2% viability after 9 h of incubation (Figure 6A). Under these conditions, nearly 50% of *rfc3-1* cells exhibited mitotic spindles, indicating that the cells entered mitosis without proper completion of DNA synthesis (our unpublished results). Microscopic observation of *rfc3-1* cells by DAPI staining showed that chromosomes were not equally distributed to the daughter cells, resulting in cell death. These results indicate that Rfc3 of *S. pombe* is required for both DNA replication and replication checkpoint.

In *S. pombe*, the checkpoint signal in S phase depends on requirement for the DNA replication, because Cut5/Rad4, for example, is involved in sending the primary signal for the DNA replication checkpoint (Saka and Yanagida, 1993; Saka *et al.*, 1994; McFarlane *et al.*, 1997). Rfc3, which is also a member of the DNA replication complex recognizing the primer–template junction, also plays a role in establishing the checkpoint signal, as shown by our results. At a permissive temperature, DNA replication is almost normal in *rfc3-1* cells, but the replication checkpoint is impaired. Thus, the roles of Rfc3 in the replication checkpoint seem to be independent of their roles in DNA replication per se. Recently, we noticed a report describing that the deletion of the *rfc2*$^+$ gene of *S. pombe* is lethal and inactivates the DNA replication checkpoint (Reynolds *et al.*, 1999), which is consistent with the result we report here for the *rfc3*$^+$ gene. In contrast, Pol ε (Cdc20), which was thought to bind to the complex composed of DNA–RFC–PCNA together with Pol δ after the RFC complex loads PCNA onto the primer terminus, lacks a function as a checkpoint sensor, although it is required for DNA replication (D'Urso and Nurse, 1997). A PCNA mutant of *S. pombe* is highly sensitive to HU, but the DNA replication checkpoint was intact in this mutant (Arroyo and Wang, 1998). Thus, among proteins required for DNA replication, Rfc proteins seem to occupy an important position in transmission of the signal to the checkpoint machinery regulating G2-M transition. In *S. cerevisiae*, it has been reported that Pol ε (encoded by *POL2*) and the associated protein Dpb11, besides being required for chromosomal replication, may also play pivotal roles in the checkpoint in response to replication blocks imposed by HU (Araki *et al.*, 1995; Navas *et al.*, 1995). Members of other replication complexes such as Rfc5 and Rfc2 are also reported to be required for the DNA replication checkpoint (Sugimoto *et al.*, 1996; Noskov *et al.*, 1998), indicating a conserved regulatory mechanism between these two yeast species, except for Pol ε of *S. pombe* as described above.

In this study, we also provided evidence that the *rfc3-1* mutant was highly sensitive to MMS and gamma irradiation and moderately sensitive to UV irradiation (Figure 6, B–D). From the nuclear and tubulin morphologies (our unpublished results), *rfc3-1* cells treated with MMS appeared to have entered mitosis without the proper checkpoint regulation, which would result in arrest at G2-M phase (Figure 7B). The results suggest that Rfc3 plays a direct role in sensing damaged DNA and transmitting the signal to the checkpoint machinery, besides being important for DNA replication and replication checkpoint function. These defects were examined and confirmed not only in mutants whose relevant genes were ectopically expressed in an *rfc3* null genetic background but also in mutants in which the relevant mutated gene was integrated (our unpublished results). This kind of triplicate function of Rfc3 of *S. pombe* was also reported for the *RFC2* and *RFC5* genes of *S. cerevisiae*, which were shown to be involved not only in DNA synthesis but also in both DNA replication and DNA damage checkpoints (Sugimoto *et al.*, 1996; Sugimoto *et al.*, 1997; Noskov *et al.*, 1998).

In *S. cerevisiae*, overexpression of *RAD24*, a budding yeast homologue of fission yeast *rad17*$^+$, suppressed the replication checkpoint defect and the DNA damage sensitivity of the *rfc5-1* mutant. Rad24 proteins were shown by immunoblot analysis to physically interact in vivo with components of the RFC complex, Rfc2 and Rfc5 (Shimomura *et al.*, 1998). Like Rad17 of fission yeast, the amino acid sequence of budding yeast Rad24 is also similar to those of the five subunits of RFC at the domains called the RFC boxes. From these results, it was suggested that *RFC5* and *RAD24* genes are related to some extent and function redundantly in the

same checkpoint pathways. In this report, we examined whether a similar relationship existed between Rfc3 and Rad17 of *S. pombe*. By reciprocal immunoprecipitation and immunoblotting experiments, we found that Rfc3 and Rad17 coimmunoprecipitated as a stable complex, suggesting their stable association in vivo (Figure 8). On the other hand, we could not observe any suppression of the sensitivity of *rfc3-1* cells to high temperature or MMS by overexpression of *rad17*$^+$ (our unpublished results). Suppression of the sensitivity to HU of *rfc3-1* cells by *rad17*$^+$ was also negligible (our unpublished results). It remains to be determined whether it is due to allele specificity.

Reduction of the phosphorylated form of Chk1 in *rfc3-1* cells suggested that the checkpoint signal emitted by Rfc3 is linked to the downstream checkpoint machinery directing G2-M arrest. However, the detailed manner in which the checkpoint signals are transmitted to these downstream checkpoint factors remains to be determined. Work is under way in our laboratory to elucidate the biochemical aspects of the signal transmission pathway through interactions with other checkpoint factors.

ACKNOWLEDGMENTS

We are indebted to A.M. Carr, T.S.-F. Wang, H. Okayama, and N.C. Walworth for the gifts of yeast strains and plasmids, K. Gull for anti-tubulin antibody (TAT-1), and K. Nabeshima for pRFC1 vector and helpful discussions. We also thank K. Tanaka and T. Nakamura for useful discussions, T. Yoneki for isolation of *rfc3*$^+$ gene, Y.A. Kishi for technical assistance, and P. Hughes for critical reading of the manuscript. This work was supported by a grant-in-aid for scientific research on priority areas from the Ministry of Education, Science, Sports and Culture of Japan and grants from the Osaka Cancer Society, The Naito Foundation, Ryoichi Naito Foundation, and Uehara Foundation.

REFERENCES

Alfa, C., Fantes, P., Hyama, J., McLeod, M., and Warbrick, E. (1993). Experiments with Fission Yeast, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Al-Khodairy, F., and Carr, A.M. (1992). DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe*. EMBO J. *11*, 1343–1350.

Al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J., Lehmann, A.R., and Carr, A.M. (1994). Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. Mol. Biol. Cell *5*, 147–160.

Apolinario, E., Nocero, M., Jin, M., and Hoffman, C.S. (1993). Cloning and manipulation of the *Schizosaccharomyces pombe his7*$^+$ gene as a new selectable marker for molecular genetic studies. Curr. Genet. *24*, 491–495.

Araki, H., Leem, S.H., Phongdara, A., and Sugino, A. (1995). Dpb11, which interacts with DNA polymerase II(epsilon) in *Saccharomyces cerevisiae*, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc. Natl. Acad. Sci. USA *92*, 11791–11795.

Arroyo, M.P., and Wang, T.S.-F. (1998). Mutant PCNA alleles are associated with cdc phenotypes and sensitivity to DNA damage in fission yeast. Mol. Gen. Genet. *257*, 505–518.

Carr, A.M. (1997). Control of cell cycle arrest by the Mec1^{sc}/Rad3^{sp} DNA structure checkpoint pathway. Curr. Opin. Genet. Dev. *7*, 93–98.

Cullmann, G., Fien, K., Kobayashi, R., and Stillman, B. (1995). Characterization of the five replication factor C genes of *Saccharomyces cerevisiae*. Mol. Cell. Biol. *15*, 4661–4671.

D'Urso, G., Grallert, B., and Nurse, P. (1995). DNA polymerase alpha, a component of the replication initiation complex, is essential for the checkpoint coupling S phase to mitosis in fission yeast. J. Cell Sci. *108*, 3109–3118.

D'Urso, G., and Nurse, P. (1997). *Schizosaccharomyces pombe cdc20*$^+$ encodes DNA polymerase epsilon and is required for chromosomal replication but not for the S phase checkpoint. Proc. Natl. Acad. Sci. USA *94*, 12491–12496.

Edwards, R.J., and Carr, A.M. (1997). Analysis of radiation-sensitive mutants of fission yeast. Methods Enzymol. *283*, 471–494.

Enoch, T., Carr, A.M., and Nurse, P. (1992). Fission yeast genes involved in coupling mitosis to completion of DNA replication. Genes & Dev. *6*, 2035–2046.

Griffiths, D.J., Barbet, N.C., McCready, S., Lehmann, A.R., and Carr, A.M. (1995). Fission yeast *rad17*: a homologue of budding yeast *RAD24* that shares regions of sequence similarity with DNA polymerase accessory proteins. EMBO J. *14*, 5812–5823.

Grimm, C., Kohli, J., Murray, J., and Maundrell, K. (1988). Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. Mol. Gen. Genet. *215*, 81–86.

Hartwell, L.H., and Weinert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629–634.

Hofmann, J.F., and Beach, D. (1994). cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. EMBO J. *13*, 425–434.

Hwang, L.H., Lau, L.F., Smith, D.L., Mistrot, C.A., Hardwick, K.G., Hwang, E.S., Amon, A., and Murray, A.W. (1998). Budding yeast Cdc20: a target of the spindle checkpoint [published erratum appears in Science 1998 May 29;280(5368):1331]. Science. *279*, 1041–1044 (erratum [1.

Kanter-Smoler, G., Knudsen, K.E., Jimenez, G., Sunnerhagen, P., and Subramani, S. (1995). Separation of phenotypes in mutant alleles of the *Schizosaccharomyces pombe* cell-cycle checkpoint gene. rad1$^+$. Mol. Biol. Cell *6*, 1793–1805.

Kelly, T.J., Martin, G.S., Forsburg, S.L., Stephen, R.J., Russo, A., and Nurse, P. (1993). The fission yeast *cdc18*$^+$ gene product couples S phase to START and mitosis. Cell *74*, 371–382.

Lindsay, H.D., Griffiths, D.J., Edwards, R.J., Christensen, P.U., Murray, J.M., Osman, F., Walworth, N., and Carr, A.M. (1998). S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe*. Genes Dev. *12*, 382–395.

Lydall, D., Nikolsky, Y., Bishop, D.K., and Weinert, T. (1996). A meiotic recombination checkpoint controlled by mitotic checkpoint genes. Nature *383*, 840–843.

Maekawa, H., Nakagawa, T., Uno, Y., Kitamura, K., and Shimoda, C. (1994). The *ste13*$^+$ gene encoding a putative RNA helicase is essential for nitrogen starvation-induced G1 arrest and initiation of sexual development in the fission *yeast Schizosaccharomyces pombe*. Mol. Gen. Genet. *244*, 456–464.

McFarlane, R.J., Carr, A.M., and Price, C. (1997). Characterization of the *Schizosaccharomyces pombe rad4/cut5* mutant phenotypes: dissection of DNA replication and G2 checkpoint control function. Mol. Gen. Genet. *255*, 332–340.

Murakami, H., and Okayama, H. (1995). A kinase from fission yeast responsible for blocking mitosis in S phase. Nature *374*, 817–819.

Navas, T.A., Zhou, Z., and Elledge, S.J. (1995). DNA polymerase epsilon links the DNA replication machinery to the S phase checkpoint. Cell *80*, 29–39.

Noskov, V.N., Araki, H., and Sugino, A. (1998). The RFC2 gene, encoding the third-largest subunit of the replication factor C complex, is required for an S-phase checkpoint in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *18*, 4914–4923.

Paulovich, A.G., and Hartwell, L.H. (1995). A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. Cell *82*, 841–847.

Reynolds, N., Fantes, P.A., and MacNeill, S.A. (1999). A key role for replication factor C in DNA replication checkpoint function in fission yeast. Nucleic Acids Res. *27*, 462–469.

Rhind, N., and Russel, P. (1998). Mitotic DNA damage and replication checkpoints in yeast. Curr. Opin. Cell Biol. *10*, 749–758.

Rowley, R., Subramani, S., and Young, P.G. (1992). Checkpoint controls in *Schizosaccharomyces pombe*: rad1. EMBO J. *11*, 1335–1342.

Saka, Y., Esashi, F., Matsusaka, T., Mochida, S., and Yanagida, M. (1997). Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1. Genes Dev. *11*, 3387–3400.

Saka, Y., Fantes, P., Sutani, T., McInerny, C., Creanor, J., and Yanagida, M. (1994). Fission yeast cut5 links nuclear chromatin and M phase regulator in the replication checkpoint control. EMBO J. *13*, 5319–5329.

Saka, Y., and Yanagida, M. (1993). Fission yeast *cut5*$^{+}$, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene. rad4^{+}. Cell *74*, 383–393.

Sambrook, J., Fritsh, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Shimomura, T., Ando, S., Matsumoto, K., and Sugimoto, K. (1998). Functional and physical interaction between Rad24 and Rfc5 in the yeast checkpoint pathways. Mol. Cell. Biol. *18*, 5485–5491.

Siede, W., Friedberg, A.S., Dianova, I., and Friedberg, E.C. (1994). Characterization of G1 checkpoint control in the yeast *Saccharomyces cerevisiae* following exposure to DNA-damaging agents. Genetics *138*, 271–281.

Siede, W., Friedberg, A.S., and Friedberg, E.C. (1993). RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *90* 7985–7989.

Sugimoto, K., Ando, S., Shimomura, T., and Matsumoto, K. (1997). Rfc5, a replication factor C component, is required for regulation of Rad53 protein kinase in the yeast checkpoint pathway. Mol. Cell. Biol. *17*, 5905–5914.

Sugimoto, K., Shimomura, T., Hashimoto, K., Araki, H., Sugino, A., and Matsumoto, K. (1996). Rfc5, a small subunit of replication factor C complex, couples DNA replication and mitosis in budding yeast. Proc. Natl. Acad. Sci. USA *93*, 7048–7052.

Tanaka, H., Tanaka, K., Murakami, H., and Okayama, H. (1999). Fission yeast *cdc24* is a replication factor C and proliferating cell nuclear antigen-interacting factor essential for S-phase completion. Mol. Cell. Biol. *19*, 1038–1048.

Tsurimoto, T., Melendy, T., and Stillman, B. (1990). Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin. Nature *346*, 534–539.

Tsurimoto, T., and Stillman, B. (1989). Purification of a cellular replication factor, RF-C, that is required for coordinated synthesis of leading and lagging strands during SV40 DNA replication in vitro. Mol. Cell. Biol. *9*, 609–619.

Uchiyama, M., Galli, I., Griffiths, D.J., and Wang, T.S. (1997). A novel mutant allele of *Schizosaccharomyces pombe rad26* defective in monitoring S-phase progression to prevent premature mitosis. Mol. Cell. Biol. *17*, 3103–3115.

Walworth, N.C., and Bernards, R. (1996). *rad*-dependent response of the *chk1*-encoded protein kinase at the DNA damage checkpoint. Science *271*, 353–356.

Weinert, T. (1998). DNA damage checkpoints update: getting molecular. Curr. Opin. Genet. Dev. *8*, 185–193.

Weinert, T.A., and Hartwell, L.H. (1988). The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science *241*, 317–322.

Willson, J., Wilson, S., Warr, N., and Watts, F.Z. (1997). Isolation and characterization of the *Schizosaccharomyces pombe rhp9* gene: a gene required for the DNA damage checkpoint but not the replication checkpoint. Nucleic Acids Res. *25*, 2138–2146.