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Rhp51-Dependent Recombination Intermediates That Do Not Generate Checkpoint Signal Are Accumulated in *Schizosaccharomyces pombe rad60* and *smc5/6* Mutants after Release from Replication Arrest

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The *Schizosaccharomyces pombe rad60* gene is essential for cell growth and is involved in repairing DNA double-strand breaks. Rad60 physically interacts with and is functionally related to the structural maintenance of chromosomes 5 and 6 (SMC5/6) protein complex. In this study, we investigated the role of Rad60 in the recovery from the arrest of DNA replication induced by hydroxyurea (HU). *rad60-1* mutant cells arrested mitosis normally when treated with HU. Significantly, Rad60 function is not required during HU arrest but is required on release. However, the mutant cells underwent aberrant mitosis accompanied by irregular segregation of chromosomes, and DNA replication was not completed, as revealed by pulsed-field gel electrophoresis. The deletion of *rhp51* suppressed the aberrant mitosis of *rad60-1* cells and caused mitotic arrest. These results suggest that Rhp51 and Rad60 are required for the restoration of a stalled or collapsed replication fork after release from the arrest of DNA replication by HU. The *rad60-1* mutant was proficient in Rhp51 focus formation after release from the HU-induced arrest of DNA replication or DNA-damaging treatment. Furthermore, the lethality of a *rad60-1 rqh1*Δ double mutant was suppressed by the deletion of *rhp51* or *rhp57*. These results suggest that Rad60 is required for recombination repair at a step downstream of Rhp51. We propose that Rhp51-dependent DNA structures that cannot activate the mitotic checkpoints accumulate in *rad60-1* cells.

Certain types of DNA damage that arise from exogenous or endogenous sources can block the progression of DNA replication (14). Blocking of DNA replication can lead to potentially lethal lesions, such as double-strand breaks (DSBs), in chromosomal DNA. DSBs can cause cell death if left unrepaired. Furthermore, inappropriate repair of these lesions results in genome instability. In eukaryotic cells, the DNA structure checkpoint responses arrest the cell cycle when DNA is damaged. DNA repair can be completed during cell cycle arrest.

In the fission yeast *Schizosaccharomyces pombe*, the DNA structure checkpoint responses require the following genes (mammalian counterparts are in parentheses): *rad3* and *rad26* (*ATR* and *ATRIP*, respectively); *rad17* (*RAD17*), encoding the clamp loader; and *rad9*, *rad1*, and *hus1* (*RAD9*, *RAD1*, and *HUS1*, respectively), encoding the 9-1-1 sliding clamp (8, 22, 37). They are involved in the activation of two downstream effector kinases, Chk1 (*CHK1*) and Cds1 (*CHK2*). Chk1 activation is required for the response to DNA damage, while Cds1 activation is required specifically in S phase. The Cds1-dependent S-phase checkpoint is required for arresting the cell cycle, stabilizing replication forks, and preventing late origin firing. The Chk1-dependent DNA damage checkpoint prevents entry into mitosis until the completion of DNA repair. There is much information in establishing the cell cycle arrest in G_2, but the mechanisms by which the checkpoint arrest is maintained and recovered are not well understood in comparison.

In eukaryotic cells, there are two major mechanisms to repair DSBs: homologous recombination and nonhomologous end joining (23, 3). In *S. pombe*, the *rad32*, *nbs1*, *rad50*, *rhp51*, *rad22*, *rhp54*, *rhp55*, and *rhp57* genes are homologous to the *Saccharomyces cerevisiae* recombination genes *MRE11*, *XRS2*, *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57*, respectively. The first three of these genes are involved in processing DSB ends, and the other genes facilitate DNA strand exchange. In addition, a recent study identified a novel pathway composed of the Swi5-Sfr1-Rhp51 alternative to Rhp55-Rhp57-Rhp51, which mediates strand exchange reactions (1).

Proteins belonging to the SMC (structural maintenance of chromosomes) superfamily also play important roles in DNA recombination repair (19, 21). SMC family proteins are structurally related to each other and contain N- and C-terminal globular nucleotide-binding domains and two central coiled-coil segments, which are separated by a central hinge region. The SMC family proteins exist as heterodimers in eukaryotes and form complexes with several non-SMC proteins. Cohesin is an SMC complex required for sister chromatid cohesion and consists of the SMC1/3 heterodimer and several non-SMC subunits. Similarly, condensin is required for the mitotic condensation of chromosomes and contains the SMC2/4 heterodimer. *S. pombe* Smc5 (Spr18) and Smc6 (Rad18) form the core of the third class of the SMC complex. To clarify the nomenclature, we referred to *rad18* in *S. pombe* as *smc6*. Recently, several non-SMC subunits of SMC5/6 were identified in *S. cerevisiae* and *S. pombe* (13, 15, 17, 32, 35, 40,

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

Strain	Genotype	Source (reference)
MP10	*h*$^-$ *leu1-32 ura4-D18*	Morishita et al. (36)
MP11	*h*$^+$ *leu1-32 ura4-D18*	Morishita et al. (36)
MPR101	*h*$^-$ *leu1-32 ura4-D18 rad60-1*	Morishita et al. (36)
MPR111	*h*$^+$ *leu1-32 ura4-D18 rad60-1*	Morishita et al. (36)
MPR121	*h*$^+$ *mat1PΔ17 leu1-32 ura4-D18 rad60-1 rhp51*::*ura4*$^+$	Morishita et al. (36)
MPR122	*h*$^+$ *mat1PΔ17 leu1-32 ura4-D18 rad60-1*	Morishita et al. (36)
MPR123	*h*$^+$ *mat1PΔ17 leu1-32 ura4-D18*	Morishita et al. (36)
MPR124	*h*$^+$ *mat1PΔ17 leu1-32 ura4-D18 rhp51*::*ura4*$^+$	Morishita et al. (36)
YSPB47	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1*	Y. Tsutsui
YSPB58	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 rhp57*::*his3*$^+$	Y. Tsutsui
chk1::*ura4*$^+$ strain	*h*$^-$ *leu1-32 ura4-D18 ade6-704 chk1*::*ura4*$^+$	A. M. Carr
cds1::*ura4*$^+$ strain	*h*$^-$ *cds1*::*ura4*$^+$	H. Okayama
rad3::*ura4*$^+$ strain	*h*$^-$ *leu1-32 ura4-D18 ade6-704 rad3*::*ura4*$^+$	A. M. Carr
TE767	*h*$^-$ *ura4-D18 rqh1*::*ura4*$^+$	T. Enoch
RH101	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 rgh1*::*ura4*$^+$	This study
RH102	*h*$^-$ *smt-0 leu1-32 ura4-D18 his3-D1 arg3-D1 rgh1*::*ura4*$^+$ *rhp57*::*his3*$^+$	This study
RH112	*h*$^-$ *smt-0 rad60-1 rhp57*::*his3*$^+$ *leu1-32 ura4-D18 his3-D1 arg3-D1*	This study
RH113	*h*$^+$ *leu1-32 ura4-D18 his3-D1 arg3-D1 rad60-1*	This study
RH114	*h*$^+$ *leu1-32 ura4-D18 his3-D1 arg3-D1 rgh1*::*ura4*$^+$	This study
RH115	*h*$^+$ *leu1-32 ura4-D18 chk1*::*ura4*$^+$ *rad60-1*	This study
RH116	*h*$^+$ *leu1-32 ura4-D18 cds1*::*ura4*$^+$ *rad60-1*	This study
RH117	*h*$^+$ *leu1-32 ura4-D18 chk1*::*ura4*$^+$ *cds1*::*ura4*$^+$	This study
rad18-74 strain	*h*$^-$ *leu1-32 ura4-D18 rad18-74*	M. J. O'Connell
RH118	*h*$^+$ *mat1PΔ17 leu1-32 ura4-D18 rhp51*::*ura4*$^+$ *rad18-74*	This study

42). The SMC5/6 complex is essential for growth. The *S. pombe* *smc6*$^+$ strain was originally identified by the analysis of a radiation-sensitive mutant, the *smc6-X* (*rad18-X*) mutant (28). A strain with another allele of *smc6*, the *smc6-74* (*rad18-74*) strain, was isolated in a genetic screening for mutants defective in DNA damage checkpoint control (44), and it was impaired in the maintenance of checkpoint arrest. Therefore, *smc6*$^+$ is suggested to be involved in both DNA repair and DNA damage checkpoint control. In *S. cerevisiae*, *SMC6* (*RHC18*) has been shown to be required for DNA damage-induced interchromosomal and sister chromatid recombination in *S. cerevisiae* (38). Recently, Torres-Rosell et al. (45) revealed that SMC5 and SMC6 are required for the proper segregation of repetitive chromosome regions.

The *rad60*$^+$ gene is essential for growth and encodes a protein involved in DSB repair through the homologous-recombination pathway (36). *rad60-1*, a temperature-sensitive mutant of *rad60*, shows intimate genetic interaction with *smc6*. Boddy et al. (6) reported that Rad60 physically interacts with the SMC5/6 complex, although the interaction is weak or transient. These studies provided strong evidence that Rad60 acts in concert with the SMC5/6 complex. Rad60 protein is phosphorylated in response to replication stress induced by hydroxyurea (HU) in a manner dependent on the Cds1 effector kinase, and this phosphorylation leads to the exit of the Rad60 protein from the nucleus (6).

To analyze the role of *rad60*$^+$ in the response to replication stress, we investigated the effects of HU on the *rad60-1* mutant. Here, we show that HU-induced replication arrest and subsequent release from the arrest cause certain chromosomal aberrations that require homologous recombination for repair and that Rad60 plays a crucial role in this repair. Rad60 is also required for preventing mitosis in response to defective repair of the replication fork, while Rhp51 is not. In the absence of functional Rad60, Rhp51-dependent initiation of homologous recombination allows cell cycle progression even though DNA repair is not accomplished.

MATERIALS AND METHODS

***S. pombe* media and methods.** *S. pombe* cells were grown in yeast extract-supplemented (YES) medium or Edinburgh minimal medium, and standard genetic and molecular procedures were employed as described previously (34). To examine sensitivity to HU on plates, serial dilutions of cells were spotted on YES medium plates containing various concentrations of HU and incubated at 26°C for 3 days. To examine sensitivity to HU in liquid medium, cells were grown in YES medium to a density of ~1×10^7/ml, and then HU was added to a concentration of 15 mM. After 0, 2, 4, 6, 8, or 10 h of incubation in the HU-containing medium, cells were appropriately diluted, spread on YES medium plates, and incubated at 30°C or 26°C for 2 to 3 days. Relative viability was determined by dividing the number of viable cells at each time point by the number of viable cells before the addition of HU (time zero).

***S. pombe* strains.** The *S. pombe* strains used in this study are listed in Table 1. A single copy of *rad60*$^+$ was integrated at the genomic *leu1* locus of MP11 or MPR111 by transformation with linearized plasmid pJK148-*rad60*$^+$.

Pulsed-field gel electrophoresis. Agarose plugs were prepared as described previously (43). Pulsed-field gel electrophoresis was carried out with 0.5% chromosomal-grade agarose (Bio-Rad) in 1× Tris-acetate-EDTA buffer by using a CHEF Mapper apparatus (Bio-Rad). The settings were as follows: 2 V/cm; pulse time, 30 min; angle, 120°; and 72 h. Gels were stained with 0.5 μg/ml ethidium bromide and photographed.

Microscopy. For visualization of nuclei and septa, cells were fixed with 70% ethanol and stained with 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) and 10 μg/ml calcofluor white, respectively. For indirect immunofluorescence, cells were fixed with 3.7% formaldehyde and processed as described previously (9). Processed cells were stained with rabbit anti-Rhp51 polyclonal antibody and Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, Oregon). Stained cells were observed under an epifluorescence microscope and photographed.

Flow cytometry. Distributions of DNA contents of the cells were analyzed by staining cells with propidium iodide and processing them for fluorescence-activated cell sorter (FACS) analysis as described in reference 41.

RESULTS

***rad60-1* cells are highly sensitive to HU.** *rad60-1* cells are temperature sensitive for growth and are hypersensitive to UV and γ rays at permissive temperatures for growth (36). As shown in Fig. 1A, *rad60-1* cells are hypersensitive to HU. To

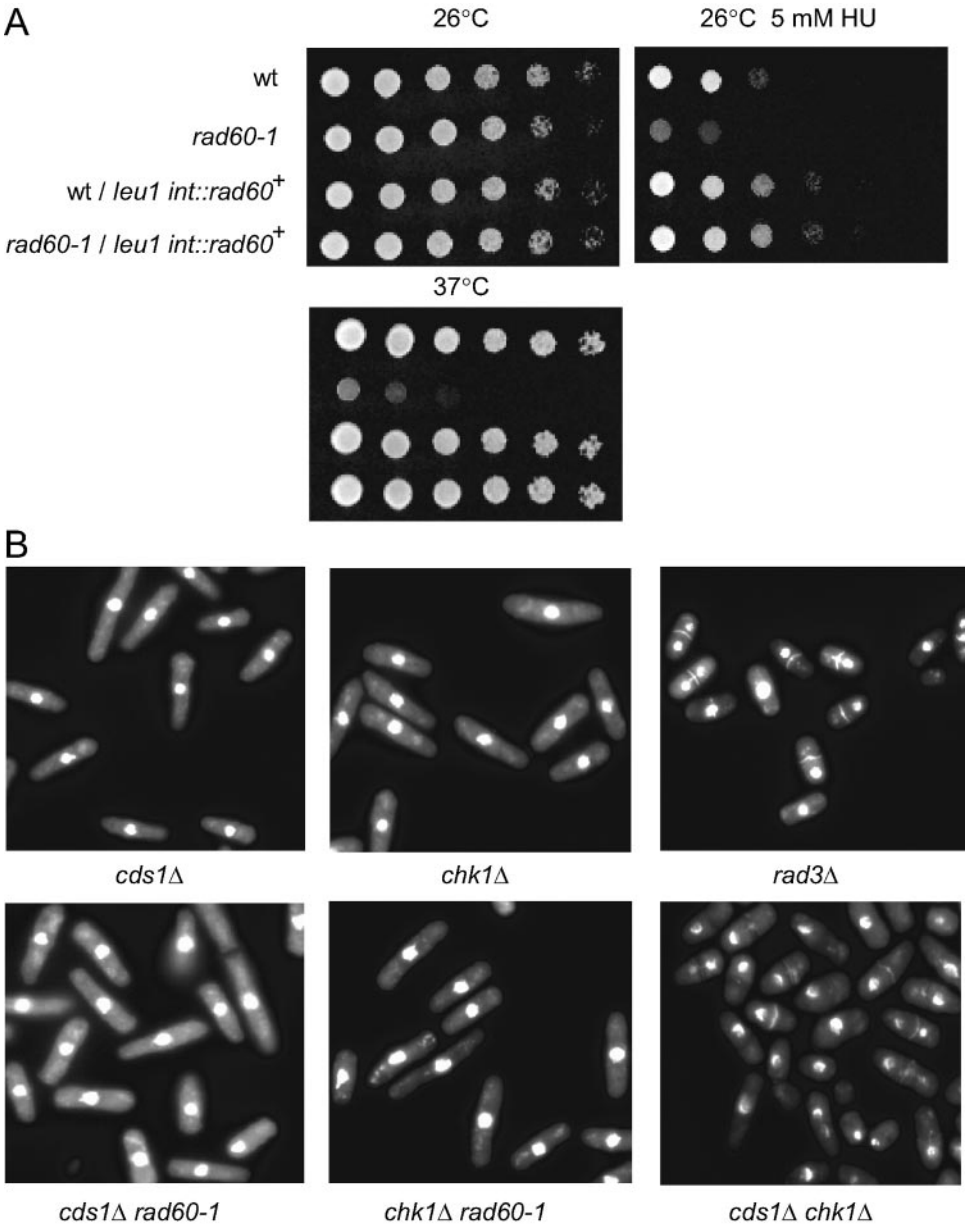

FIG. 1. *rad60-1* cells are sensitive to HU and proficient in arresting cell cycle progression in response to HU. (A) Spot assays to examine HU and the temperature sensitivities of *rad60-1* cells. *leu1 int*::*rad60*$^+$ represents the *rad60*$^+$ gene integrated at the *leu1* locus. Five serial dilutions of the indicated strains were spotted on YES medium plates with or without HU and incubated at the indicated temperatures for 3 days. (B) Analysis of cell cycle checkpoint. Cells of the indicated mutants were incubated in YES medium containing 15 mM HU at 30°C for 4 h. Cells were fixed with 70% ethanol and stained with DAPI and calcofluor white. wt, wild type.

examine whether the sensitivity of the *rad60-1* strain is complemented by *rad60*$^+$, we introduced the single-copy *rad60*$^+$ gene into the *leu1* locus of the wild-type or *rad60-1* strain. Both of the transformants were resistant to HU and grew normally at a high temperature of 37°C. Thus, *rad60-1* is a recessive mutation with respect to HU and temperature sensitivity. This indicates that *rad60-1* is a hypomorphic allele.

***rad60-1* cells are proficient in Cds1-dependent cell cycle arrest in response to HU.** Since Rad60 is phosphorylated in a Cds1-dependent manner (6), we examined whether *rad60-1* cells can properly undergo mitotic arrest when they are treated with HU. HU treatment causes wild-type cells to undergo Cds1-dependent cell cycle arrest. When *cds1*Δ cells are treated with HU, the Chk1-dependent DNA damage checkpoint is activated and the cells undergo cell cycle arrest before entry into mitosis (5, 30). Therefore, Cds1-dependent cell cycle arrest can be assayed in a *chk1*Δ background. *cds1*Δ *chk1*Δ cells septated, and chromosomes segregated aberrantly by being cultured in the presence of HU-like *rad3*Δ cells, which lack both Cds1- and Chk1-dependent checkpoint activation (Fig. 1B). In contrast, *rad60-1 chk1*Δ cells could arrest cell cycle progres-

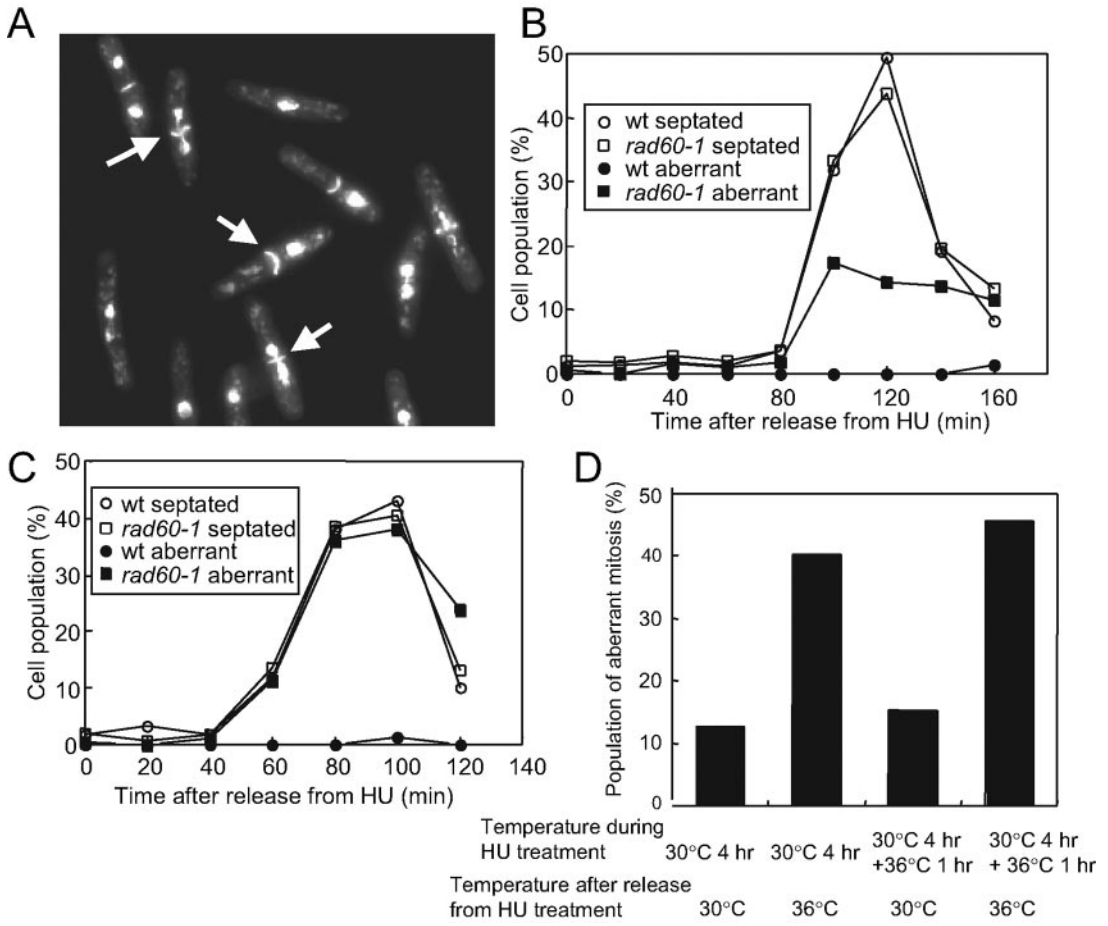

FIG. 2. Rad60's function is required for normal chromosome segregation after release from HU treatment. (A) Aberrant mitosis of *rad60-1* cells after release from HU treatment. Exponentially growing wild-type and *rad60-1* cells were incubated in YES medium containing 15 mM HU at 30°C for 4 h, released into fresh YES medium without HU, and further incubated at 30°C for 2 h. Cells were stained with DAPI and calcofluor white after fixation. Arrows indicate cells undergoing aberrant mitosis. (B, C, and D) Frequency of aberrant mitosis of *rad60-1* and wild-type cells. (B) Exponentially growing wild-type and *rad60-1* cells were incubated in YES medium containing 15 mM HU at 30°C for 4 h, released into fresh YES medium without HU, and further incubated at 30°C. Wild-type or *rad60-1* cells that septated and were undergoing aberrant mitosis were counted at 20-min intervals. (C) Wild-type or *rad60-1* cells were treated as in panel B, except that they were incubated at 36°C after release from HU. (D) *rad60-1* cells were incubated in YES medium containing 15 mM HU at 30°C for 4 h, with or without subsequent incubation at 36°C for 1 h. The cells were then released into HU-free medium and incubated at 30°C or 36°C for 120 min. wt, wild type.

sion in response to HU and became elongated by being cultured in the presence of HU (Fig. 1B). This indicates that *rad60-1* cells can normally activate Cds1 kinase in the presence of HU. In addition, *cds1*Δ *rad60-1* double mutant cells could arrest the cell cycle in response to HU (Fig. 1B), indicating that *rad60-1* cells can normally activate the Chk1-dependent DNA damage checkpoint. *rad60-1 cds1*Δ *chk1*Δ cells septated by being cultured in the presence of HU-like *cds1*Δ *chk1*Δ cells (data not shown), indicating that the arrest of the *rad60-1* cells in the presence of HU depends on the presence of either *cds1* or *chk1*.

***rad60*$^+$ is required for normal mitosis after release from arrest by HU.** We next investigated the morphology of *rad60-1* cells after release from the arrest by HU. After release from HU, *rad60-1* cells entered aberrant mitosis in which septation occurred without proper chromosome segregation (Fig. 2A and B). Cells containing nuclear material bisected by a septum and cells with unequal segregation of chromosomes were frequently observed. To examine whether Rad60 is required during replication stress or after recovery from it, we examined the effect of temperature shift before and after release from HU. Since *rad60-1* is recessive with respect to temperature sensitivity (Fig. 1A), the Rad60-1 protein is suggested to lose its functions at the restrictive temperature. Thus, by inactivating Rad60 before or after release from HU and monitoring the frequency of subsequent aberrant mitosis, we can distinguish whether the function of Rad60 is required before or after release from HU for subsequent normal mitosis. The percentage of *rad60-1* cells undergoing aberrant mitosis was significantly increased by shifting to the restrictive temperature after release from HU (Fig. 2C). At the restrictive temperature, more than 90% of septated cells entered aberrant mitosis. When *rad60-1* cells synchronized at G_2 phase were incubated at the restrictive temperature, only 6% of the cells entered aberrant mitosis during the first cell cycle (data not shown).

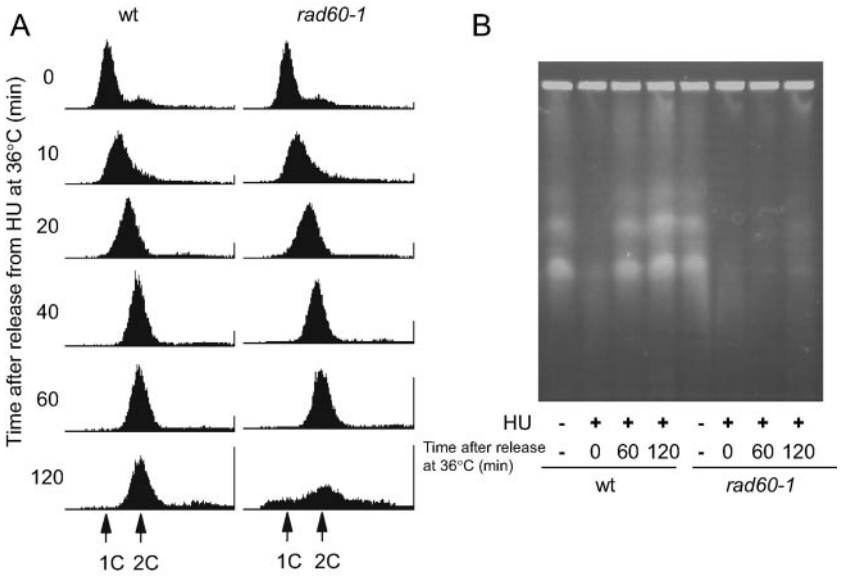

FIG. 3. DNA synthesis and aberrant DNA structure after release from HU arrest. Exponentially growing wild-type and *rad60-1* cells were incubated in YES medium containing 15 mM HU for 4 h at 30°C. Cells were then incubated at 36°C after release from HU treatment. (A) FACS analysis of DNA contents of *rad60-1* cells after release from HU treatment. Cells were fixed at the indicated time points, stained with propidium iodide, and analyzed by FACS to determine the DNA content. (B) Analysis of chromosomes of *rad60-1* cells after release from HU treatment. Genomic DNA was subjected to pulsed-field gel electrophoresis. Samples were taken at the indicated time points. wt, wild type.

Therefore, the high frequency of aberrant mitosis seen after release from HU is not likely to be due merely to an effect of high-temperature incubation of the mutant in S phase. In contrast, incubation at the restrictive temperature before HU release did not increase aberrant mitosis (Fig. 2D). Under these conditions, wild-type cells entered normal mitosis with proper chromosome segregation (data not shown). These results suggest that the Rad60 function is required for a process after release from replication arrest by HU and that lack of this function leads to abnormal chromosome segregation in the next mitosis. We could not find a requirement for the Rad60 function during arrest by HU for normal chromosome segregation in these experiments. Interestingly, wild-type and *rad60-1* cells formed septa with the same timing after the removal of HU when they were grown at 36°C, even though *rad60-1* cells entered into aberrant mitosis while wild-type cells underwent normal mitosis (Fig. 2B and C).

Formation of aberrant DNA structure in *rad60-1* cells after release from their arrest by HU. Although *rad60-1* cells stop cell division in the presence of HU and initiate cell division with the same timing as wild-type cells after the removal of HU, they undergo aberrant mitosis. This suggests that these cells contain unreplicated or unrepaired DNA. To examine whether *rad60-1* cells can complete bulk DNA synthesis, we examined the DNA contents of *rad60-1* cells after their release from HU. As shown in Fig. 3A, wild-type and *rad60-1* cells could resume and complete bulk DNA synthesis with similar kinetics even at the restrictive temperature. In both strains, most cells contained 2C DNA until 40 min after their release. This result indicates that *rad60*$^+$ is not required for bulk DNA synthesis after release from the arrest by HU. At 120 min, *rad60-1* cells exhibited a broadened peak of DNA content as revealed by FACS analysis, consistent with aberrant chromosome segregation. We next examined chromosomal DNA by pulsed-field gel electrophoresis (Fig. 3B). In wild-type cells, three distinct chromosomal bands disappeared in the presence of HU and were recovered within 1 h after the release of the cells from HU. In contrast, the chromosomal bands of *rad60-1* cells did not recover even after 2 h, at which time point most cells entered mitotic stage (Fig. 2C). Therefore, most of the chromosomal DNA of *rad60-1* cells under these conditions probably remained in the wells and may have consisted of DNA with branched structures like replicating DNA forks or recombination intermediates. Mild but similar effects were observed when *rad60-1* cells were incubated at 30°C after being released from HU (data not shown). This suggests that *rad60-1* cells have not completed replication or have undergone incomplete recombination when they enter the next mitosis after release from the arrest of DNA replication by HU. It is also possible that restarting of replication occurs but results in or leaves behind a structure that is aberrant.

Checkpoint arrest in *rad60-1* cells after their release from HU is restored by the deletion of *rhp51*. To examine the effects of Rhp51-dependent homologous recombination on the response of *rad60-1* cells to HU, we next performed the same experiments as those described above under an *rhp51*Δ background. As shown in Fig. 4A, *rhp51*Δ and *rhp51*Δ *rad60-1* cells showed almost the same sensitivity to HU. Chromosomal bands in either mutant mostly did not recover within 2 h after the release from HU (Fig. 4B). This suggests that *rhp51* is required for the normal restarting of replication after release from arrest by HU. Meister et al. (33) have shown that *rhp51*Δ cells complete bulk DNA synthesis after their release from HU. *rhp51*Δ *rad60-1* cells also completed bulk DNA synthesis after their release from HU, as shown by FACS analysis (data not shown). Therefore, it is possible that recombination-

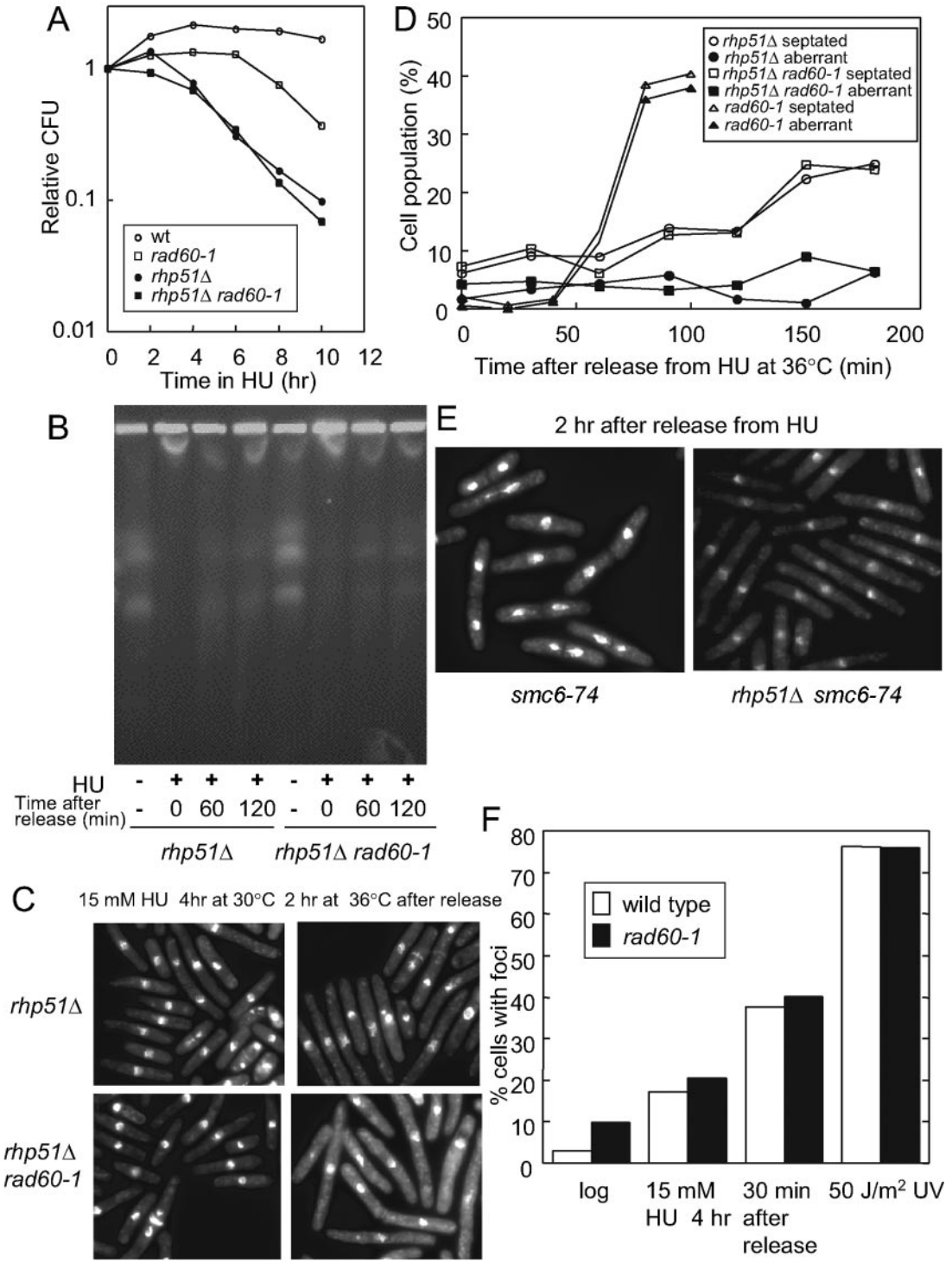

FIG. 4. Phenotypes of *rad60-1* cells in response to HU treatment are dependent on Rhp51 function. (A) Sensitivity of *rad60-1* and *rhp51*Δ cells to HU treatment. Exponentially growing cells of the indicated strains were incubated in YES medium containing 15 mM HU at 30°C. Samples were taken at 2-h intervals. Relative numbers of CFU were calculated as described in Materials and Methods. (B) Analysis of chromosomes of *rhp51*Δ and *rhp51*Δ *rad60-1* cells. Genomic DNA was subjected to pulsed-field gel electrophoresis. Samples were taken at the indicated time points after release from 15 mM HU treatment at 30°C. (C) Cellular morphology of *rhp51*Δ and *rhp51*Δ *rad60-1* cells. *rhp51*Δ and *rhp51*Δ *rad60-1* cells were fixed after the indicated treatments and stained with DAPI and calcofluor white. (D) Time course analysis of septum formation and aberrant mitosis of *rad60-1* and *rhp51*Δ cells. Exponentially growing *rhp51*Δ and *rhp51*Δ *rad60-1* cells were incubated in YES medium containing 15 mM HU at 30°C for 4 h, released into fresh YES medium without HU, and further incubated at 36°C. *rhp51*Δ or *rhp51*Δ *rad60-1* cells that were septated or undergoing aberrant mitosis were counted at 30-min intervals. The results for the *rad60-1* single mutant were the same as those shown in Fig. 2C. (E) Aberrant mitosis of *smc6-74* cells after release from HU treatment. Exponentially growing *smc6-74* and *sm6-74 rhp51*Δ cells were incubated in YES medium containing 15 mM HU at 30°C for 4 h, released into fresh YES medium without HU, and further incubated at 30°C for 2 h. Cells

dependent processes are required for the accurate completion of DNA replication after arrest by HU even though they are not necessarily required for bulk DNA synthesis. Interestingly, both *rad60*$^+$ and *rad60-1* cells were highly elongated after their release from HU in the *rhp51*Δ background (Fig. 4C). Time course analysis revealed a significant delay of entry into mitosis in these cells (Fig. 4D). In addition, entry into aberrant mitosis was not enhanced by the *rad60-1* mutation under this background. These results indicate that the function defective in the *rad60-1* mutant is not required for the maintenance of checkpoint arrest or normal chromosome segregation in the absence of *rhp51*$^+$. Since Rad60 functions in recombination repair in concert with the SMC5/6 complex, we asked whether a mutation in a component of the SMC5/6 complex causes defects similar to those of the *rad60-1* mutation. *smc6-74* cells also underwent aberrant mitosis after their release from HU (Fig. 4E). As in the case of *rad60-1* cells, the deletion of *rhp51* suppressed the entry of the mutant cells into aberrant mitosis (Fig. 4E). A mutation in *nse4* (*rad62*), encoding a subunit of SMC5/6 (35, 40), also caused aberrant mitosis after release from HU (data not shown), indicating that this is a common phenotype caused by defects in the SMC5/6 complex and Rad60 functions. Since Rhp51 focus formation is an indication of recombination function in vivo, we next examined the formation of Rhp51 foci after cells were released from HU treatment. In both wild-type and *rad60-1* cells, Rhp51 foci were formed predominantly in S phase, 30 min after the release from HU (Fig. 4F), and the majority of cells contained less than three foci per nucleus (data not shown). On the other hand, UV irradiation at 50 J/m^2 induced a higher proportion of cells containing Rhp51 foci, and most of the focus-containing cells had more than three foci. This may suggest that only a small number of the replication forks require Rhp51 for restart from arrest by HU. In either case, the proportions of cells with Rhp51 foci were comparable in wild-type and *rad60-1* cells, suggesting that *rad60-1* cells can initiate homologous recombination normally in response to induced DNA damage. These results suggest that Rad60 is required for recombination repair after release from arrest by HU at a step after the formation of Rhp51 foci.

***rad60-1* cells enter Rhp51-dependent aberrant mitosis after UV irradiation in G_2 phase.** The results above show that Rhp51-dependent initiation of recombination repair at recovery from the arrest of DNA replication forks causes aberrant mitosis in *rad60-1* cells. Rhp51 is known to be required for resistance to UV in G_2 phase (31). In this case, the DNA damage checkpoint pathway prevents entry into the next mitosis (2). To examine whether the Rhp51-dependent aberrant mitosis seen in *rad60-1* cells was specific for recovery from HU, cells were synchronized in G_2 and irradiated with UV. *rad60-1* cells could arrest the cell cycle in response to UV irradiation and entered the next mitosis with kinetics similar to those of wild-type cells even though a significant portion of *rad60-1* cells underwent aberrant mitosis (Fig. 5A). In contrast, the *rhp51*Δ mutation inhibited entry into mitosis of *rad60-1* mutant cells, and the mutant cells became highly elongated after UV irradiation (Fig. 5B and C). These results indicate that the entry of *rad60-1* cells into Rhp51-dependent aberrant mitosis is not only caused by DNA damage induced by replication stress but also induced by UV in G_2 phase.

Mutations in the homologous-recombination genes involved in the strand exchange reaction suppress the HU sensitivity of *rqh1*Δ cells and the synthetic lethality of the *rqh1*Δ *rad60-1* double mutant. *S. pombe* Rqh1 is suggested to be required for recombination repair at a step downstream of Rhp51 (27), and *rqh1*Δ cells show morphological phenotypes similar to those of *rad60-1* cells after their release from arrest by HU (12, 44). We next examined whether the phenotypes of *rqh1*Δ cells are also dependent on Rhp51. As shown in Fig. 6A, the HU sensitivity of *rqh1*Δ cells was partially suppressed by *rhp57*Δ or *rhp51*Δ. *rqh1*Δ cells underwent either aberrant mitosis or cell elongation 120 min after their release from arrest by HU (Fig. 5B) and differed from the *rad60-1* mutant cells, which underwent mostly aberrant mitosis (Fig. 2A). The appearance of *rqh1*Δ mutant cells that underwent aberrant mitosis was suppressed by *rhp51*Δ (Fig. 6B). *rad60-1* cells are synthetically lethal with *rqh1*Δ, similar to *rad60-3* cells (6). This synthetic lethality was partially suppressed by *rhp57*Δ (Fig. 6C). Some spores of the *rqh1*Δ *rad60-1* double mutant formed almost-invisible tiny colonies, and none of them grew after restreaking. *rhp57*Δ *rqh1*Δ *rad60-1* cells formed small colonies (Fig. 6C), and they grew after restreaking, although more slowly than their parental strains (data not shown). Viable spores of the *rhp51*Δ *rqh1*Δ *rad60-1* triple mutant were also isolated from a cross between *rhp51*Δ *rad60-1* and *rqh1*Δ cells, although total spore viability was extremely low (data not shown). From these results, we conclude that the lethality of the *rqh1*Δ *rad60-1* double mutant can be suppressed by a deficiency in the initiation of homologous recombination.

DISCUSSION

Recombination functions are required for recovery from the arrest of DNA replication by HU treatment. In this study, we analyzed the effects of *rad60-1*, *rhp51*Δ, and *rqh1*Δ mutations on the phenotypes of cells after their release from the arrest of DNA replication caused by HU. HU is an inhibitor of ribonucleotide reductase, and hence HU treatment results in deoxynucleoside triphosphate (dNTP) starvation. In response to dNTP depletion, cells stop replicating DNA, firing late replication origins, and entering mitosis. After the removal of HU, cells restart DNA replication (11, 25, 39). In this study, we showed that *rhp51* is required for normal replication after release from arrest by HU (Fig. 4B). It is known that when replication is stalled by obstacles such as damaged DNA, proteins tightly bound to DNA, or some secondary structures of

were stained with DAPI and calcofluor white after fixation. (F) Proportion of Rhp51 focus-forming cells after HU and UV treatment. Exponentially growing cells, cells incubated in YES medium containing 15 mM HU 4 h at 30°C, cells incubated in YES medium containing 15 mM HU 4 h at 30°C and released in HU-free medium at 36°C for 30 min, and cells irradiated with 50 J/m^2 UV and incubated at 30°C 1 h were processed for indirect immunofluorescence using anti-Rhp51 antibody. The number of cells containing Rhp51 foci was counted and divided by the number of total cells to calculate the percentage. wt, wild type.

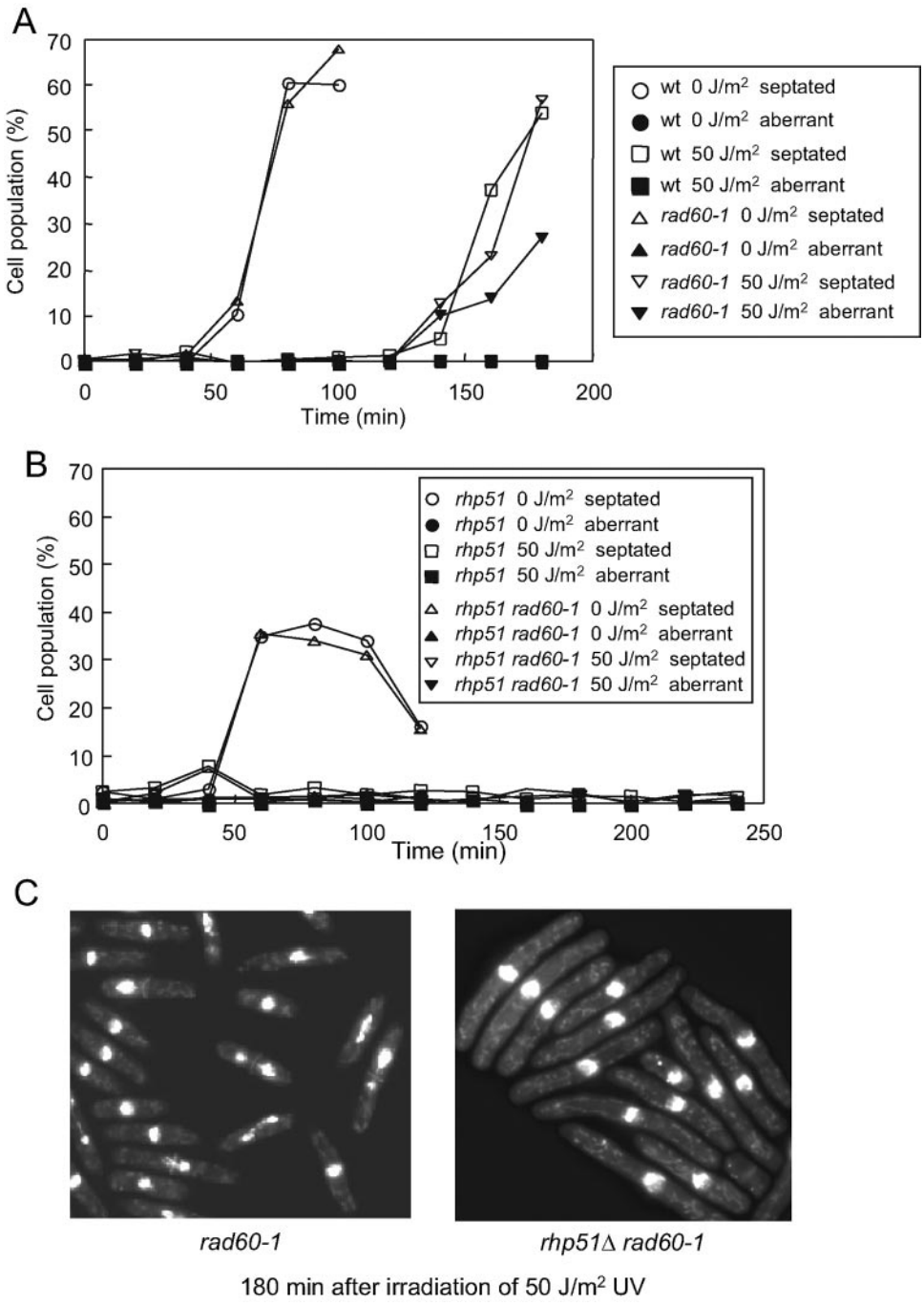

FIG. 5. *rad60-1* cells enter Rhp51-dependent aberrant mitosis after UV irradiation in G_2. Wild-type and *rad60-1* cells (A) and *rhp51*Δ and *rhp51*Δ *rad60-1* cells (B) were synchronized in G_2 by a lactose gradient procedure and irradiated with 50 J/m^2 UV or mock irradiated. Cultures were incubated at 30°C. Cells that septated and were undergoing aberrant mitosis were counted at 20-min intervals. (C) Cells stained with DAPI and calcofluor white after fixation at the indicated time point were photographed. wt, wild type.

DNA, the replication fork is regenerated in a manner dependent on homologous-recombination machinery (10). In the case of release from arrest by HU, replication is stalled merely by dNTP depletion, and there should be no obstacles to be overcome after the removal of HU. It is possible that replication forks frequently fail to maintain the integrity of the replication machinery and collapse during the arrest and that the cells require recombination-dependent fork regeneration for a restart of replication. Chk1-dependent cell cycle delay and transient Chk1 phosphorylation after recovery from HU arrest have been reported (7), and this is consistent with the possible presence of stalled or collapsed replication forks that require recombination for restarting replication. Recently, Bjergbaek et al. (4) showed that *S. cerevisiae* Rad51 is required for DNA polymerase

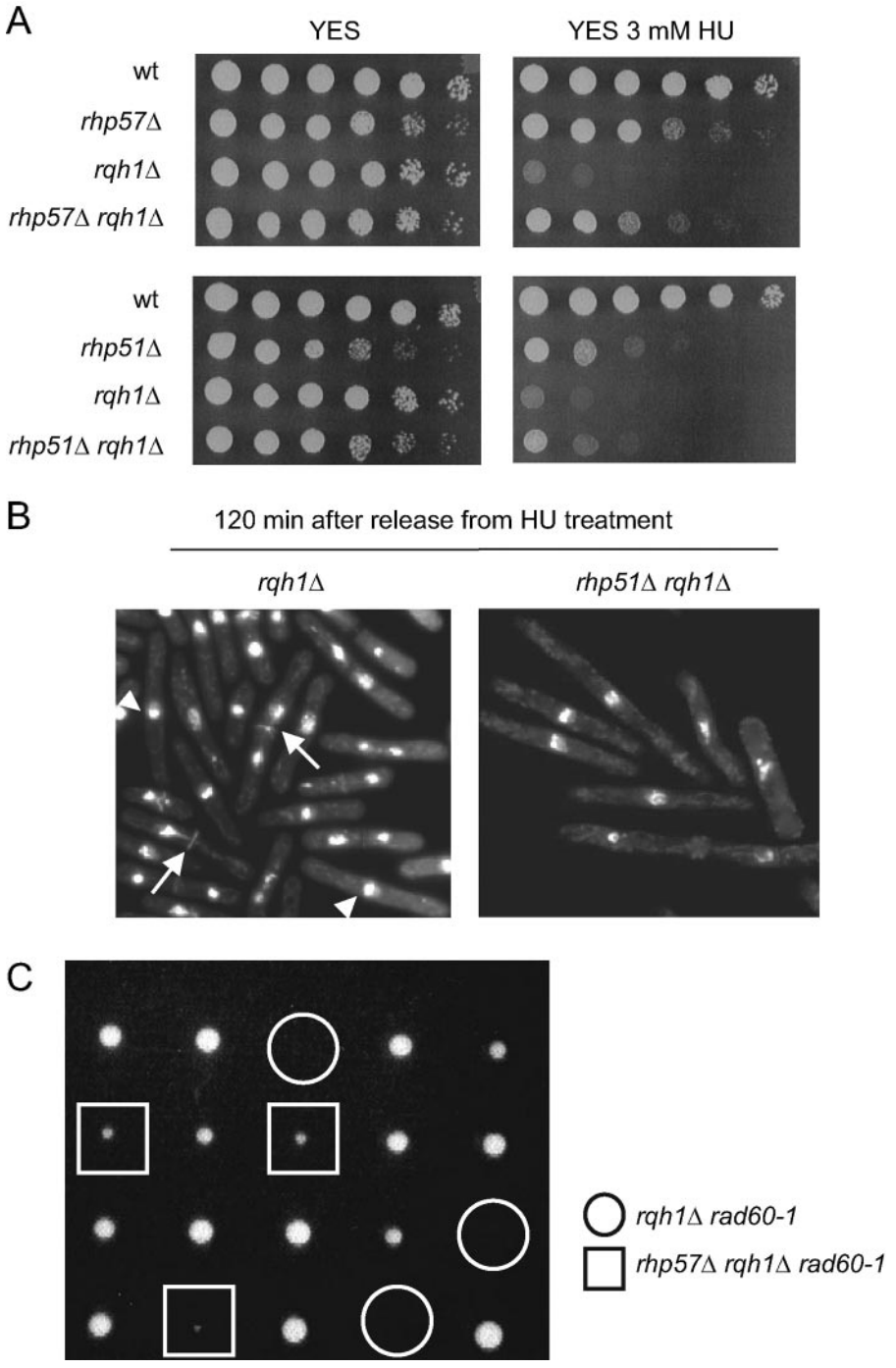

FIG. 6. *rqh1*Δ cells show phenotypes similar to but distinct from those of *rad60-1* cells. (A) Sensitivity to HU. Spot assay to show HU sensitivities of the indicated strains. Plates were incubated at 30°C for 2 days. (B) Cellular morphology of *rqh1*Δ and *rhp51*Δ *rqh1*Δ cells after release from HU treatment. *rqh1*Δ and *rhp51*Δ *rqh1*Δ cells were incubated for 120 min after release from HU treatment for 4 h, fixed, and stained with DAPI and calcofluor white. Arrows and arrowheads indicate cells undergoing aberrant mitosis and cells undergoing elongation without mitosis, respectively. (C) Suppression of the lethality of *rqh1*Δ *rad60-1* cells by the deletion of *rhp57*. *rhp57*Δ *rad60-1* and *rqh1*Δ cells were crossed, and the resulting spores were subjected to tetrad analysis. Dissected spores were grown at 26°C for 5 days on YES medium plates. Segregants enclosed by circles and squares indicate *rqh1*Δ *rad60-1* and *rhp57*Δ *rqh1*Δ *rad60-1* cells, respectively. wt, wild type.

ε stabilization at replication forks stalled by HU. They proposed that Rad51-dependent homologous recombination promotes the resolution of regressed replication forks without cleavage.

Rad60 is required for the recombination repair of replication forks after release from the arrest by HU. Rad60 functions in the Rhp51 pathway of recombination repair in concert with the SMC5/6 complex (6, 36). We showed that *rad60* is required for normal replication after recovery from arrest by HU (Fig. 3B). Temperature shift experiments showed that the Rad60 function is required for a process after release from cell cycle arrest

by HU and probably not for initiating or maintaining arrest by HU (Fig. 2C and D). This is consistent with the role of Rad60 and Rhp51 in recombination repair because Rhp51 foci are formed predominantly after release from HU treatment (Fig. 4F). Meister et al. (33) showed that Rad22 forms nuclear foci after release from HU treatment. They showed that homologous recombination is required after release from HU treatment and is repressed during arrest by HU. *rad60-1* cells could resume and carry out bulk DNA synthesis with kinetics similar to those of wild-type cells after removal of the HU, as revealed by FACS analysis (Fig. 3A).

Rad60 and Smc5/6 function in recombination repair at a step after the formation of the Rhp51 foci. Rad60 appears to work at a step of the recombination repair after the formation of the Rhp51 foci, because *rhp51* is epistatic to *rad60-1* with respect to sensitivity to UV, γ rays, and HU (36) (Fig. 4A), and *rad60-1* cells can form Rhp51 foci normally in response to HU removal or exposure to UV (Fig. 4F). Recently, Torres-Rosell et al. revealed that X-shaped DNAs are accumulated in *S. cerevisiae smc6* mutants at the rRNA gene locus and that the deletion of *RAD52* partially suppresses the temperature sensitivity of the mutant (45). Consistent with these results, our results suggest that Smc5/6 and Rad60 are required for the proper resolution of sister chromatid junctions formed by the action of *rad51* (*rhp51*).

rad60-1 cells enter mitosis after recovery from arrest by HU with the same time course as that of wild-type cells, even though the mitosis in the *rad60-1* cells is aberrant (Fig. 2A and C). The timing of reentry into mitosis after their release from arrest by HU is regulated by the Chk1-dependent DNA damage checkpoint (7). Since *chk1*Δ cells enter the next mitosis earlier than wild-type cells after release from HU (7), *rad60-1* cells appear to be proficient in the activation and maintenance of the Chk1-dependent DNA damage checkpoint. On the other hand, *rhp51*Δ cells are delayed with respect to reentry into mitosis (Fig. 4C and D). The reason for this difference can be explained as follows. In the *rhp51*Δ cells, stalled or collapsed replication forks are not processed by the Rhp51-dependent recombination pathway, and hence the checkpoint control mechanisms keep inhibiting mitosis. In *rad60-1* cells, stalled or collapsed forks are processed by the Rhp51 protein into a form that escapes detection by the checkpoint systems. However, the recombination intermediates are not further processed properly because of the defect in Rad60's function, the chromosomes are not properly segregated, and the cells undergo aberrant mitosis. The *rad60-1 rhp51*Δ double mutant behaves like the *rhp51*Δ single mutant (Fig. 4), and this observation is consistent with the above-described model. Possibly, the recombination intermediates that accumulate in the *rad60-1* cells do not trigger the checkpoint signal, while strand breaks or single-strand DNA regions not processed by Rhp51 do.

rad60-1 cells underwent aberrant mitosis after exposure to UV in G_2 phase, with kinetics similar to those of wild-type cells which enter normal mitosis (Fig. 5A), and entry into aberrant mitosis was suppressed by the deletion of *rhp51* (Fig. 5B). In this case, there is no replication fork and cells enter the next mitosis without entry into S phase. These results suggest that Rad60 is also required for the recombination repair of DNA damage other than stalled or collapsed replication forks at a step downstream of Rhp51 and that the model described above is applicable to this case. Verkade et al. (46) showed that *smc6-74* cells enter mitosis with kinetics similar to those of wild-type cells without completion of the repair of DNA damage induced by γ rays. In addition, when cells are exposed to UV after depletion of the Smc6 protein, cells enter aberrant mitosis (16), as *rad60-1* cells do (Fig. 5). The kinetics of entry into mitosis are almost the same as those of wild-type cells in this case. These observations can also be explained by this model. *smc6-74* and *nse4-1* (*rad62-1*) cells showed phenotypes very similar to that of *rad60-1* cells after their release from arrest by HU (Fig. 4E and data not shown). Therefore, they are common phenotypes of cells defective in the Rad60 or SMC5/6 function.

Rad60 and Rqh1 cooperate in recombination repair. Rqh1 is the *S. pombe* ortholog of *Escherichia coli* RecQ, *S. cerevisiae* Sgs1, and mammalian RecQ family helicases, such as Wrn, Blm, and Rts. The RecQ family helicases are thought to be involved in the processing of recombination intermediates (reviewed in references 18, 24, and 26). Recently, Wu and Hickson (47) showed that BLM and human TOPO IIIα catalyze the resolution of a recombination intermediate containing a double Holliday junction. The *rqh1*Δ mutant undergoes aberrant mitosis after release from HU treatment (Fig. 6B) (44), and this is consistent with the above-described model, in which some kinds of recombination intermediates cause abnormal chromosome segregation. However, the *rqh1*Δ mutant differs from the *rad60-1* mutant in that the *rqh1*Δ cells undergo either aberrant mitosis or cell elongation without mitosis after their release from HU (Fig. 6B), while the majority of *rad60-1* cells undergo aberrant mitosis (Fig. 2A). Laursen et al. (27) reported evidence suggesting that Rqh1 acts both upstream and downstream of Rhp51. Probably, the cells which entered aberrant mitosis suffered from failure in the processing of recombination intermediates, while the elongated cells suffered from a defect in an earlier event in recombination. We showed that the *rqh1*Δ *rad60-1* double mutation is lethal and that this lethality is suppressed by loss of the recombination gene *rhp51* or *rhp57*. This suggests that Rqh1 and Rad60 play roles in preventing the accumulation of some kind(s) of recombination intermediate(s). Possibly, Rqh1 and Rad60 have roles in the resolution and/or prevention of the formation of recombination intermediates during vegetative growth. An Rqh1 homologue in *E. coli*, RecQ, was suggested to play a role in recruiting RecA, an Rhp51 homologue, to a stalled replication fork and preventing the formation of chicken foot structures (20). *rqh1*Δ cells show higher sensitivity to HU than *rhp51*Δ or *rhp57*Δ cells, and this sensitivity is partially suppressed by the deletion of *rhp51* or *rhp57* (Fig. 6A), suggesting that some kind of DNA structure which arises from Rhp51-dependent initiation of recombination repair is more toxic to cells than DNA damage unprocessed by Rhp51. Our results described here suggest that Rad60, the SMC5/6 complex, and Rqh1 are required for processing such DNA structures, which fail to generate the signal of the DNA damage checkpoint and consequently cause abnormal chromosome segregation. Recently, Liberi et al. (29) showed that in *S. cerevisiae sgs1*Δ cells, Rad51-dependent DNA structures accumulate at damaged replication forks and that the apparent failure of the proper activation of Rad53 is suppressed by the deletion of *rad51*.

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