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The *Schizosaccharomyces pombe rad11*$^+$ Gene Encodes the Large Subunit of Replication Protein A

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Replication protein A (RPA) is a heterotrimeric single-stranded DNA-binding protein present in all eukaryotes. In vitro studies have implicated RPA in simian virus 40 DNA synthesis and nucleotide excision repair, but little direct information is available about the in vivo roles of the protein. We report here the cloning of the largest subunit of RPA (*rpa1*$^+$) from the fission yeast *Schizosaccharomyces pombe*. The *rpa1*$^+$ gene is essential for viability and is expressed specifically at S phase of the cell cycle. Genetic analysis revealed that *rpa1*$^+$ is the locus of the *S. pombe* radiation-sensitive mutation *rad11*. The *rad11* allele exhibits pleiotropic effects consistent with an in vivo role for RPA in both DNA repair and DNA synthesis. The mutant is sensitive to both UV and ionizing radiation but is not defective in the DNA damage-dependent checkpoint, consistent with the hypothesis that RPA is part of the enzymatic machinery of DNA repair. When incubated in hydroxyurea, *rad11* cells initially arrest with a 1C DNA content but then lose viability coincident with reentry into S phase, suggesting that DNA synthesis is aberrant under these conditions. A significant fraction of the mutant cells subsequently undergo inappropriate mitosis in the presence of hydroxyurea, indicating that RPA also plays a role in the checkpoint mechanism that monitors the completion of S phase. We propose that RPA is required to maintain the integrity of replication complexes when DNA replication is blocked. We further suggest that the *rad11* mutation leads to the premature breakdown of such complexes, thereby preventing recovery from the hydroxyurea arrest and eliminating a signal recognized by the S-phase checkpoint mechanism.

The integrity of the eukaryotic genome is maintained in part by mechanisms that ensure the fidelity of DNA replication and mediate the repair of DNA damage. Two classes of genes involved in these mechanisms have been identified by genetic experiments. One class encodes proteins that carry out the enzymatic steps in DNA replication and repair. The second class is involved in coordinating these processes with other cell cycle events. The products of the latter genes comprise surveillance mechanisms (or checkpoints) that act to delay cell cycle progression until DNA replication and/or repair are completed (40, 49). The checkpoint mechanism that monitors DNA replication normally prevents the cell from entering mitosis when DNA synthesis is blocked by inhibitors such as hydroxyurea. Similarly, the DNA damage checkpoint(s) causes mitotic delay following UV or ionizing radiation.

A large number of radiation-sensitive mutants of the fission yeast *Schizosaccharomyces pombe* have been isolated and characterized (37). Most are defective in the enzymatic pathways responsible for repairing lesions in the DNA. However, mutants in seven linkage groups (*rad1*, *rad3*, *rad9*, *rad17*, *rad26*, *hus1*, and *chk1*) are competent to repair DNA damage but fail to arrest in G_2 following irradiation, indicating that they are defective in the DNA damage checkpoint (2, 44). Interestingly, mutants in six of the groups are also unable to arrest in S phase in response to inhibitors of DNA replication, suggesting that there is considerable overlap of the DNA damage and S-phase checkpoints (2, 3, 16, 21, 44). The overlap is not complete, however, since one mutant (*chk1* strain) is defective exclusively in the DNA damage checkpoint (26, 48).

The signals recognized by the DNA damage and S-phase checkpoints are not known. One reasonable possibility is that they recognize specific structural features of the intermediates in the DNA replication or repair reactions. If this is the case, it would not be surprising to find that some of the components of the enzymatic machinery of replication and/or repair are required for the generation and maintenance of the checkpoint signal. The recent observation that certain mutants of the gene encoding DNA polymerase ε of the budding yeast *Saccharomyces cerevisiae* are defective in the S-phase checkpoint is consistent with this possibility (42).

Replication protein A (RPA; also known as replication factor A and human single-stranded DNA-binding protein) was first identified in human cells as one of the cellular factors essential for simian virus 40 (SV40) DNA replication in vitro (18, 51, 52). RPA is a single-stranded DNA-binding protein comprised of three tightly associated subunits of 70, 32, and 14 kDa in human cells. The large subunit of RPA (RPA-70) contains the single-stranded DNA-binding activity (6, 17). Homologous 70-kDa subunits have been identified in a variety of eukaryotic species (1, 6, 7, 17, 22). The other two subunits of RPA are essential for DNA replication, but their precise functions are not known (17, 27).

Biochemical studies in the SV40 DNA replication system have demonstrated that RPA plays a central role in the initiation and elongation of DNA chains. It interacts with the SV40 initiator protein, T antigen, and facilitates the initial unwinding of the SV40 origin of replication (11, 14, 35, 51, 52). The unwinding of the origin is probably the rate-determining step in initiation, and RPA is the only cellular protein known to be required for this step. RPA also promotes the priming and elongation of new DNA chains via direct interactions with

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DNA polymerase α/primase and T antigen (11, 14, 35). The presence of RPA in the initiation complex during the critical first steps raises the possibility that it has a role in regulating the initiation of DNA replication. In addition to its role(s) in DNA replication, RPA is probably involved in other cellular transactions involving DNA. The protein has been shown to be essential for nucleotide excision repair in vitro (12). RPA also stimulates the activity of eukaryotic homologous pairing proteins in vitro, suggesting that it may be involved in recombination (22, 37).

Studies with the budding yeast *S. cerevisiae* have demonstrated that the genes for all three of the RPA subunits (*RFA1*, *RFA2*, and *RFA3*) are essential and are coordinately expressed at the G_1/S phase boundary (5). A number of temperature-sensitive mutations in the *RFA1* gene encoding the large subunit of yeast RPA display an extended S phase and plasmid instability indicative of defects in DNA replication as well as an increased sensitivity to UV radiation, suggesting impairment of DNA repair mechanisms (30). Mutations in the *RFA1* gene also have significant effects on the frequency of mitotic recombination in vivo (19, 30, 47).

In this report, we describe the cloning and characterization of the *S. pombe* RPA-70 gene (designated *rpa1*$^+$). Physical mapping studies revealed that *rpa1*$^+$ resides near the locus of the radiation-sensitive *rad11* mutant. We demonstrated by linkage analysis that *rpa1*$^+$ is allelic to *rad11* and that plasmids carrying *rpa1*$^+$ rescue the radiation sensitivity of the *rad11A* strain. Characterization of the *rad11A* strain revealed a number of interesting phenotypes not previously observed due to the presence of an extragenic suppressor mutation in the original strain *rad11.404*. These phenotypes, which segregate as a single locus, include a reduced growth rate, sensitivity to temperature and the DNA synthesis inhibitor hydroxyurea, and an increased rate of meiotic recombination. Although the *rad11A* strain is sensitive to both UV and ionizing radiation, it is not defective in the DNA damage checkpoint, consistent with the hypothesis that RPA is part of the enzymatic machinery of DNA repair. Studies with hydroxyurea suggest that RPA is also required to maintain the integrity of the replication complex when DNA replication is blocked. The mutant cells rapidly lose viability during recovery from the hydroxyurea block and eventually undergo an aberrant cell division. Thus, our data indicate that RPA has important roles in DNA replication, repair, and recombination in vivo. Although the protein is not required for the DNA damage checkpoint, we propose that it may play an important role in maintaining the signal recognized by the S-phase checkpoint.

MATERIALS AND METHODS

Yeast strains and methods. All *S. pombe* strains used (Table 1) are isogenic to the wild-type *h*$^-$ strain 972. Media, growth conditions, and genetic manipulations were as previously described (38). For all temperature shift experiments carried out in liquid culture, cells were grown at 25°C and then shifted to the nonpermissive temperature of 36°C. For experiments done on solid media, incubation was done at 30°C as the permissive temperature and shifted to 36°C. When cells were grown on plates, there was no difference in phenotype at 25 or 30°C. Unless otherwise noted, cells were transformed by electroporation as previously described (26). Cells were fixed in 70% ethanol for staining with 4′,6-diamidino-2-phenylindole (DAPI) (38). Flow cytometry (fluorescence-activated cell sorting [FACS]) was performed as previously described (46), using a Becton Dickinson FACScan.

Cloning the *rpa1*$^+$ gene. Degenerate PCR primers were designed based on alignments of known RPA1 sequences. RPA1-1 was a 37-mer with a *Bam*HI restriction site (5′-CGGGATCCGSIGMIATHMGIGCIACIGYNTTYAAYGA-3′), and RPA1-3 was a 28-mer with a *Hin*dIII restriction site (5′-CCCAAGCT TKYRTYRCAYTTYTCRCANC-3′), where S = G or C, M = A or C, Y = C or T, K = G or T, R = A or G, and H = A, C, or T. PCRs were carried out in a 100-μl volume containing 10 μM each primer, 5 U of AmpliTaq polymerase (Perkin-Elmer), and appropriate buffer conditions. The PCR (30 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 2 min followed by 1 cycle of 72°C for 7 min) was carried out with 100 ng of *S. pombe* wild-type genomic DNA as template and resulted in a single band of the predicted size. The fragment was digested and subcloned into pBluescript KS$^-$ (Stratagene), creating plasmid pAR4. DNA sequencing of the insert revealed significant homology to the known RPA1 sequences. The PCR fragment was also used as a probe to screen an *S. pombe* genomic library in the shuttle vector pUR18N. Four unique clones were identified, one of which contained the complete *rpa1*$^+$ gene plus approximately 1 kb of flanking sequence on each side.

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

Strain	Genotype
TK7	*h*$^-$ *leu1-32 ura4-D18 ade6-M210*
TK8	*h*$^+$ *leu1-32 ura4-D18 ade6-M216*
TK26	*h*$^+$ *cdc25-22 ura4-D18*
YRC8	*h*$^+$/*h*$^-$ *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 his7-366/his7-366*
rad11.404	*h*$^+$ *rad11.404 ura4-D18*
rad11A	*h*$^+$ *rad11A ura4-D18*
APY2	*h*$^-$ *rad11A ura4-D18 leu1-32*
APY6	*h*$^+$ *rad11A::*(*rpa1*$^+$ *ura4*$^+$) *ura4-D18*
APY13	*h*$^+$ *rad11A ura4-D18 leu1-32 ade6-M216*
APY15	*h*$^-$ *rad11A ura4-D18 leu1-32 ade6-M210*
APY43	*h*$^+$ *rad11A::*(*rpa1*$^+$ *ura4*$^+$) *ura4-D18 leu1-32 ade6-M210*
APY44	*h*$^+$ *rpa1*$^+$*::*(*rpa1*$^+$ *ura4*$^+$) *ura4-D18 leu1-32 ade6-M216*
APY45	*h*$^-$ *rad11A::*(*rpa1*$^+$ *ura4*$^+$) *ura4-D18 leu1-32 ade6-M216*

Physical mapping. A cosmid library of overlapping genomic clones gridded on a high-density nylon filter was provided by E. Maier (24, 34). The pAR4 insert described above was used as a probe to screen the library. Six positive clones were identified, and analysis of their coordinates indicated that the *rpa1*$^+$ gene resided at one end of chromosome II.

Disruption and integration of the *rpa1*$^+$ gene. To disrupt the *rpa1*$^+$ gene, a derivative of the genomic clone was obtained in which the *rpa1*$^+$ coding sequence was largely replaced with the *ura4*$^+$ marker. The 3′ *Bam*HI-*Hin*dIII *rpa1*$^+$ genomic fragment was subcloned into pBluescript, creating plasmid pAEP1. The 5′ sequence of *rpa1*$^+$ from nucleotide positions +1 to +807 was generated by PCR using one internal primer and a 5′ primer containing an *Xho*I site. The PCR product was digested with *Xho*I and *Hin*dIII (generating a fragment containing *rpa1* +1 to +717) and inserted into the *Xho*I-*Hin*dIII sites of pAEP1, creating pRC60. This plasmid was cleaved with *Hin*dIII, and the entire *ura4*$^+$ gene was inserted as a *Hin*dIII fragment, creating pRC61.

The *Xho*I-*Bam*HI fragment derived from pRC61 was used to create a disruption of the *rpa1*$^+$ gene. The fragment was transformed using lithium acetate (38) into an *h*$^+$/*h*$^-$ diploid (YRC8) which can be maintained by intragenic complementation of the two *ade6* markers. Transformants with a stable *ura4*$^+$ marker were sporulated, and tetrad analysis was carried out. Disruption of the *rpa1*$^+$ gene was confirmed by Southern blot hybridization and genomic PCR analysis on DNA prepared from transformants which produced two viable and two inviable spores.

The *S. pombe* strains carrying a *ura4*$^+$ marker linked to *rpa1*$^+$ were constructed as follows. Plasmid pAEPR1.9 carrying the *rpa1*$^+$ gene and the *ura4*$^+$ marker was cleaved at the unique *Nhe*I site. The resulting linear DNA fragment was introduced into haploid *S. pombe* strains, and stable integrants with *ura4*$^+$ inserted at the *rpa1*$^+$ locus were verified by Southern blot analysis.

Synchronized cell populations. The *cdc25-22* strain (TK26) was grown at 25°C in minimal medium to a density of 5×10^6 cells/ml and then shifted to the nonpermissive temperature (36°C) for 6 h to obtain a population arrested in G_2. The culture was rapidly cooled to 25°C and returned to 25°C for growth. Samples were taken every 15 min to score septation index and prepare RNA as previously described (38).

Counterflow centrifugal elutriation was carried out with a Beckman JE-6B elutriation rotor and a J2-21 centrifuge. Approximately 5×10^9 cells were loaded at a rotor speed of 4,000 rpm and a pump speed of 4 ml/min. The cells were equilibrated for 20 min and then recovered by increasing the pump speed by increments of 0.5 ml/min. The smallest G_2 cells came out of the rotor at a pump speed of 5.5 ml/min. The average yield was 2×10^8 cells, approximately 4% of the input material. Cells were then diluted into fresh medium as required.

Irradiation. UV irradiation was carried out by using a Stratagene Stratalinker UV source. A known density of log-phase cells was plated onto selective medium, incubated at 30°C for 30 min, and then subjected to the indicated dose of UV radiation. Gamma irradiation was carried out by using a Gammacell 1000 ^{137}Cs source (6.2 Gy/min). Cells from a logarithmic culture were diluted to a density of 10^4 cells/ml and exposed to the gamma source. Aliquots were then plated onto selective medium. Plates were incubated at 30°C, and colonies were counted after 72 h. Survival was expressed as a percentage of the number of colonies formed on unirradiated plates.

To determine radiation checkpoint competence, cells were synchronized in G_2 phase on a 7.5 to 30% lactose gradient as previously described (4). Samples were subjected to various doses of ionizing radiation and incubated at 30°C. Aliquots were removed every 15 min and fixed in ethanol as described above. Samples were stained with DAPI, and the percentage of cells passing through mitosis was scored by comparing the proportion of mononucleate G_2 cells with the number of mitotic, binucleate, and septated cells.

Meiotic recombination assay. The frequency of meiotic intragenic recombination between two point mutations within the *ade6* gene (*ade6-M210* and *ade6-M216*) was assayed in wild-type and mutant backgrounds. h^- *ade6-M210* strains were crossed to h^+ *ade6-M216* strains. Spores were plated onto supplemented Edinburgh minimal medium (EMM; Bio 101) and EMM-adenine plus phloxin B. Phloxin B was added to ensure that only haploid colonies are scored as the *ade6* mutations can complement each other in a diploid. Colonies were counted after 7 days of incubation at 30°C. The number of viable spores was then compared to the number of ade^+ recombinants. Each cross was carried out at least five times, and the average frequency of recombination was determined.

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been assigned GenBank accession number U75446.

RESULTS

Cloning and characterization of *S. pombe rpa1*$^+$. The gene encoding the large subunit of *S. pombe* RPA was cloned using degenerate PCR amplification. The design of the PCR primers was based upon alignment of RPA1 protein sequences from human, *Xenopus*, and budding yeast cells (1, 7, 17, 22). One pair of degenerate primers yielded a reaction product of the predicted size, and subsequent sequence analysis confirmed that it was homologous to RPA1 of other species. This PCR product was used as a probe to screen an *S. pombe* genomic library. One of four unique clones identified (pAEPR1.9) contained the complete *rpa1*$^+$ gene plus flanking regions (Fig. 1). Sequence analysis indicated that the *rpa1*$^+$ gene contains two exons (total of 1,827 nucleotides) and a single 69-nucleotide intron with appropriate consensus splicing signal sequences (45). The presence of the intron was confirmed by cloning and sequencing the *rpa1*$^+$ cDNA. The *rpa1*$^+$ open reading frame encodes a predicted protein of 609 amino acids with a molecular mass of 68.2 kDa, similar in size to the Rpa1 subunits of human cells and budding yeast (5, 17). Amino acid sequence comparisons revealed that the *S. pombe* Rpa1 sequence is 38% identical to the human RPA1 and 39% identical to the *S. cerevisiae* Rpa1. Purification of the *S. pombe* RPA and cloning of the three *rpa*$^+$ genes has also recently been reported by another group (25).

Examination of the 5′ untranslated region of the gene revealed several exact and near matches to the MCB (*Mlu*I cell cycle box) motif ACGCGT (32). MCB sequences are the recognition elements for the transcription factor complex DSC1, which has been shown to activate the transcription of several *S. pombe* genes at the beginning of S phase (23, 26, 32). The DSC1 complex contains the Cdc10 and Res1/Sct1 proteins, which are required for the transition from G_1 into S phase. The most highly conserved MCB sequence in the *rpa1*$^+$ gene is at −92 relative to the ATG start codon.

To determine whether *rpa1*$^+$ transcription is periodic in the cell cycle, the level of *rpa1*$^+$ mRNA was monitored in a population of temperature-sensitive *cdc25* cells synchronized by release from G_2 arrest. Measurement of the septation index showed that the population was highly synchronous during the first division cycle and somewhat less so during the second division cycle (Fig. 2). Total RNA recovered at the indicated times was analyzed for *rpa1*$^+$ mRNA as well as for two control mRNAs derived from the *cdc22*$^+$ and *cdc2*$^+$ genes. The *cdc22*$^+$ gene is a known target of the DSC1 transcription factor and is expressed periodically, while the *cdc2*$^+$ gene is expressed at a constant level during the cell cycle (32). The level of *rpa1*$^+$ mRNA is clearly periodic, increasing sharply just prior to the peak of septation, which is concomitant with S phase, and decreasing rapidly thereafter. The pattern of expression of the *rpa1*$^+$ mRNA is quite similar to that of the *cdc22*$^+$ mRNA, suggesting that *rpa1*$^+$ transcription may be regulated by DSC1 via the MCB elements. The G_1/S-phase-specific transcription of *rpa1*$^+$ is typical of other replication genes and suggests a requirement for the gene in DNA replication (23, 26, 32). Preliminary data indicate that Rpa1 protein levels do not vary significantly during the cell cycle (data not shown), suggesting that the protein has a relatively long half-life. This observation is consistent with results of Rpa1 protein analysis in human cells (13).

Physical mapping and disruption of *S. pombe rpa1*$^+$. Physical mapping of genes in *S. pombe* has been greatly facilitated by the production of ordered genomic DNA libraries (24, 34, 36). The *rpa1*$^+$ degenerate PCR product described above was used as a probe to screen an ordered cosmid library provided by E. Maier (24, 34). The probe hybridized to six clones, all localized within a small region at one end of chromosome II near the region assigned to *rad11*. The localization of the *rpa1*$^+$ gene was further confirmed by DNA sequence analysis of the genomic clone pAEPR1.16. This analysis revealed that *rpa1*$^+$ is adjacent to *mik1*$^+$, which had previously been localized to the same region of chromosome II (33).

To determine whether *rpa1*$^+$ is an essential gene, a null allele of the gene was generated. For this purpose, one of the two chromosomal copies of *rpa1*$^+$ in an *S. pombe* diploid strain was largely replaced by the *ura4*$^+$ gene. After confirmation of the replacement by Southern blot analysis and genomic PCR, the resulting heterozygous diploid was sporulated and subjected to tetrad analysis. A minimum of eight tetrads were examined in each of three independent isolates, and in all cases there were two viable and two inviable spores. All viable spores were auxotrophic for uracil, indicating that deletion of the *rpa1*$^+$ gene is lethal (data not shown). Most of the null mutant spores divided only once, and none of the disruptants gave rise to more than three cells. Although the terminal phenotypes that we observed were not uniform, a significant fraction of the *RPA1*-disrupted cells were considerably longer than normal.

Phenotypic characterization of *rad11*. The *S. pombe rad11.404* strain has been shown to be sensitive to both UV and gamma radiation (43). The close proximity of *rpa1*$^+$ to *rad11* suggested the possibility that the two are allelic. Before testing this possibility, we backcrossed the *rad11* strain (*rad11.404*) to a wild-type *S. pombe* strain. Spores derived from the backcross were plated on rich medium containing phloxin B, a stain that accumulates in dead cells. Three phenotypes were observed: lightly staining wild-type colonies (45%), intermediate-staining colonies (26%), and darkly staining colonies (29%). Clones representative of each phenotype were picked and compared to the parental strains with respect to both UV and gamma radiation sensitivity. The intermediate-staining clones and the parental *rad11.404* strain were equally sensitive to UV or gamma irradiation. The darkly staining colonies were more sensitive to both UV and gamma irradiation than the parental *rad11.404* (data not shown). We concluded that *rad11.404* harbors an extragenic suppressor of the radiation sensitivity phenotype. A strain derived from the darkly staining colonies, which lacked this suppressor, was designated *rad11A* and was used for all our subsequent experiments. The suppressor was not characterized further. A comparison of the gamma radiation sensitivities of *rad11*$^+$, *rad11.404*, and *rad11A* is presented in Fig. 3A. The *rad11.404* strain displays radiation sensitivity intermediate to that of *rad11*$^+$ and *rad11A* strains at all doses examined.

In addition to radiation sensitivity, the *rad11A* mutant ex-

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-248   act act att ctt ttc agc ttt taa ggg ctt ttc tat agc aat
-206   gtg ttg tgc gca aag gat aac ttt gaa agt aaa caa cca ttg
-164   ttg aca tgc acg tac aca aac aac aca tct taa agt tac gcg
-122   taa tat taa aat gtg tta cga cgc gtc atg acg cgt tcg cgt
-80    ttt tca ttt ggg ata acg cga cgc ggc ttc gtt ctt ttg cct
-38    taa acc aac cac taa tta aaa aaa aaa gaa ttt gca aac ATG
                                                        M
+4     GCT GAG CGA TTA TCC GTG GGT GCA CTT CGT ATA ATC AAg taa
       A   E   R   L   S   V   G   A   L   R   I   I   N
+46    gtt aac tgt cca aat tat ata ctc aaa aga ttt cga caa att

+88    ggg atg tta aca cgt ttc ttt agT ACA TCC GAT GCT TCG TCT
                                      T   S   D   A   S   S
+130   TTT CCT CCT AAT CCA ATT CTC CAA GTC TTA ACC GTG AAG GAG
       F   P   P   N   P   I   L   Q   V   L   T   V   K   E
+172   CTA AAC TCA AAT CCA ACT TCT GGT GCT CCT AAA AGA TAT CGT
       L   N   S   N   P   T   S   G   A   P   K   R   Y   R
+214   GTT GTC CTT TCC GAT TCA ATC AAT TAT GCG CAA TCT ATG TTA
       V   V   L   S   D   S   I   N   Y   A   Q   S   M   L
+256   AGC ACC CAG CTG AAC CAT TTG GTT GCC GAA AAT AAA CTT CAA
       S   T   Q   L   N   H   L   V   A   E   N   K   L   Q
+298   AAA GGA GCG TTC GTA CAG CTT ACC CAA TTC ACT GTA AAC GTT
       K   G   A   F   V   Q   L   T   Q   F   T   V   N   V
+340   ATG AAA GAA AGA AAA ATT TTG ATT GTT CTT GGT TTG AAT GTT
       M   K   E   R   K   I   L   I   V   L   G   L   N   V
+382   TTA ACT GAA CTT GGC GTA ATG GAT AAA ATT GGT AAT CCC GCT
       L   T   E   L   G   V   M   D   K   I   G   N   P   A
+424   GGT TTA GAA ACA GTT GAC GCT TTA CGG CAG CAG CAA AAT GAG
       G   L   E   T   V   D   A   L   R   Q   Q   Q   N   E
+466   CAA AAC AAT GCT AGC GCA CCA CGA ACA GGT ATT TCG ACA AGC
       Q   N   N   A   S   A   P   R   T   G   I   S   T   S
+508   ACT AAC TCA TTT TAT GGA AAC AAT GCT GCA GCT ACA GCA CCG
       T   N   S   F   Y   G   N   N   A   A   A   T   A   P
+550   GCT CCA CCT CCC ATG ATG AAG AAG CCA GCA GCG CCG AAT AGC
       A   P   P   P   M   M   K   K   P   A   A   P   N   S
+592   CTT AGC ACA ATA ATC TAC CCA ATT GAA GGT CTC TCT CCA TAT
       L   S   T   I   I   Y   P   I   E   G   L   S   P   Y
+634   CAG AAT AAA TGG ACT ATT CGT GCC CGT GTT ACT AAC AAA TCT
       Q   N   K   W   T   I   R   A   R   V   T   N   K   S
+676   GAG GTT AAA CAT TGG CAT AAT CAA CGA GGT GAA GGA AAG CTT
       E   V   K   H   W   H   N   Q   R   G   E   G   K   L
+718   TTT AGC GTA AAT TTA CTC GAT GAA AGT GGG GAG ATT CGT GCA
       F   S   V   N   L   L   D   E   S   G   E   I   R   A
+760   ACA GGC TTT AAT GAC CAG GTT GAT GCT TTT TAT GAT ATC TTA
       T   G   F   N   D   Q   V   D   A   F   Y   D   I   L
+802   CAG GAA GGT TCG GTT TAT TAT ATA TCT CGT TGT CGT GTG AAC
       Q   E   G   S   V   Y   Y   I   S   R   C   R   V   N
+844   ATT GCA AAA AAA CAA TAT ACC AAT GTT CAA AAC GAG TAT GAG
       I   A   K   K   Q   Y   T   N   V   Q   N   E   Y   E
+886   CTG ATG TTT GAA CGT GAT ACT GAA ATA AGG AAA GCT GAG GAT
       L   M   F   E   R   D   T   E   I   R   K   A   E   D
+928   CAA ACT GCC GTC CCC GTT GCG AAG TTT AGT TTT GTT TCT CTA
       Q   T   A   V   P   V   A   K   F   S   F   V   S   L
+970   CAA GAA GTC GGC GAT GTT GCC AAA GAT GCT GTT ATC GAT GTA
       Q   E   V   G   D   V   A   K   D   A   V   I   D   V
+1012  ATT GGT GTA CTC CAA AAC GTT GGA CCA GTT CAA CAA ATT ACC
       I   G   V   L   Q   N   V   G   P   V   Q   Q   I   T

+1054  AGT CGT GCA ACT TCT CGT GGA TTT GAC AAG CGT GAT ATC ACT
       S   R   A   T   S   R   G   F   D   K   R   D   I   T
+1096  ATC GTT GAC CAA ACA GGA TAT GAA ATG CGT GTT ACG CTT TGG
       I   V   D   Q   T   G   Y   E   M   R   V   T   L   W
+1138  GGA AAG ACA GCT ATT GAA TTC TCT GTT TCT GAA GAA AGT ATT
       G   K   T   A   I   E   F   S   V   S   E   E   S   I
+1180  CTC GCT TTC AAA GGA GTT AAA GTT AAT GAC TTT CAA GGA CGC
       L   A   F   K   G   V   K   V   N   D   F   Q   G   R
+1222  TCT TTG TCA ATG TTA ACT AGC AGC ACC ATG TCG GTA GAT CCA
       S   L   S   M   L   T   S   S   T   M   S   V   D   P
+1264  GAT ATC CAG GAA TCT CAT TTG TTA AAA GGT TGG TAT GAT GGA
       D   I   Q   E   S   H   L   L   K   G   W   Y   D   G
+1306  CAG GGT AGA GGG CAG GAA TTT GCC AAA CAT AGC GTA ATT TCA
       Q   G   R   G   Q   E   F   A   K   H   S   V   I   S
+1348  TCT ACT TTA TCT ACT ACT GGA AGG AGT GCT GAA CGT AAA AAT
       S   T   L   S   T   T   G   R   S   A   E   R   K   N
+1390  ATT GCT GAG GTT CAA GCC GAA CAT CTG GGA ATG TCA GAA ACA
       I   A   E   V   Q   A   E   H   L   G   M   S   E   T
+1432  CCC GAT TAT TTT AGC TTA AAG GGC ACC ATT GTT TAC ATT CGA
       P   D   Y   F   S   L   K   G   T   I   V   Y   I   R
+1474  AAG AAA AAC GTG TCA TAT CCT GCT TGC CCT GCT GAT TGC
       K   K   N   V   S   Y   P   A   C   P   A   A   D   C
+1516  AAC AAA AAG GTT TTT GAT CAA GGT GGC TCC TGG CGA TGT GAG
       N   K   K   V   F   D   Q   G   G   S   W   R   C   E
+1558  AAA TGT AAT AAA GAA TAT GAT GCT CCT CAA TAC CGT TAT ATT
       K   C   N   K   E   Y   D   A   P   Q   Y   R   Y   I
+1600  ATA ACT ATT GCT GTA GGT GAT CAT ACG GGA CAA CTC TGG CTC
       I   T   I   A   V   G   D   H   T   G   Q   L   W   L
+1642  AAC GTC TTT GAC GAC GTC GGT AAA TTA ATC ATG CAT AAA ACG
       N   V   F   D   D   V   G   K   L   I   M   H   K   T
+1684  GCA GAC GAA TTG AAT GAT CTA CAA GAA AAC GAC GAA AAT GCC
       A   D   E   L   N   D   L   Q   E   N   D   E   N   A
+1726  TTT ATG AAT TGT ATG GCA GAA GCT TGC TAT ATG CCA TAT ATC
       F   M   N   C   M   A   E   A   C   Y   M   P   Y   I
+1768  TTT CAA TGT CGT GCT AAG CAA GAC AAT TTT AAA GGT GAA ATG
       F   Q   C   R   A   K   Q   D   N   F   K   G   E   M
+1810  CGA GTA CGC TAT ACA GTG ATG TCA ATT AAT CAA ATG GAC TGG
       R   V   R   Y   T   V   M   S   I   N   Q   M   D   W
+1852  AAG GAA GAG TCT AAA AGA TTG ATA AAT TTC ATT GAG TCT GCT
       K   E   E   S   K   R   L   I   N   F   I   E   S   A
+1894  CAA taa aac att gtg ttt tac gtc tcg ctg gtc gag taa ttg
       Q
+1936  agt ttt tgg ctt aaa tcg cga aaa cta cga ttt atg att ttg
+1978  cat aca ttg att aca aag tga ctc ctt tcc tta tga ttt ggt
+2020  act tca ctt aca ttt aac tag att taa agt aat tat ata tga
+2062  tct gaa ttt ttg gag taa tta gtt ctg ttg ctt aca tat taa
+2104  agt tcc ttg atg ggt aat tta cgc tat aat ggc ttg att gaa
+2146  atc acc gct aaa aga atg caa tga tat ctt tta ata tga agt
+2188  a
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FIG. 1. Nucleotide sequence of *S. pombe rpa1*$^+$ and flanking genomic regions. The deduced amino acid sequence is given in single-letter code, and the intron and flanking sequences are indicated by lowercase nucleotides. MCB-like elements present in the 5′ untranslated region are underlined (32).

hibited a number of phenotypes which had been masked by the presence of the extragenic suppressor. The growth rate of *rad11A* was markedly lower than that of the wild type, with a doubling time of approximately 6 h, compared to 3.5 h for wild-type cells. The strain was also temperature sensitive for growth and profoundly sensitive to hydroxyurea (Fig. 3B). Analysis of logarithmically growing cells by flow cytometry revealed that most of the mutant cells had a DNA content of approximately 2C. Although the distribution of cellular DNA content was somewhat broader than that observed for wild-type cells, no distinct subpopulations of G_1- or S-phase cells were observed (data not shown).

Further genetic analysis demonstrated that all of the phenotypes of the *rad11A* strain were the result of mutation of a single gene. The *rad11A* strain was crossed to a wild-type strain and subjected to tetrad analysis. All tetrads yielded two wild-type and two radiation-sensitive spores. The radiation-sensitive spores invariably exhibited reduced growth rate and sensitivity to hydroxyurea (data not shown). Thus, all phenotypes segregated as a single Mendelian locus.

***rpa1*$^+$ and *rad11*$^+$ are allelic.** A restriction map of the *rpa1*$^+$ locus is presented in Fig. 4A. To examine the relationship between *rpa1*$^+$ and *rad11*$^+$, we first transformed the mutant strain with plasmids containing each of the four *rpa1*$^+$ genomic clones and assayed the transformants for radiation sensitivity. Of the four plasmids, only pAEPR1.9, which contained the full-length *rpa1*$^+$ gene, was capable of rescuing the UV and gamma radiation sensitivity of *rad11A* (Fig. 3B and 4B). Integration of pAEPR1.9 in single copy at the *rad11* locus was also sufficient to rescue the UV and gamma sensitivity (Fig. 5A). In

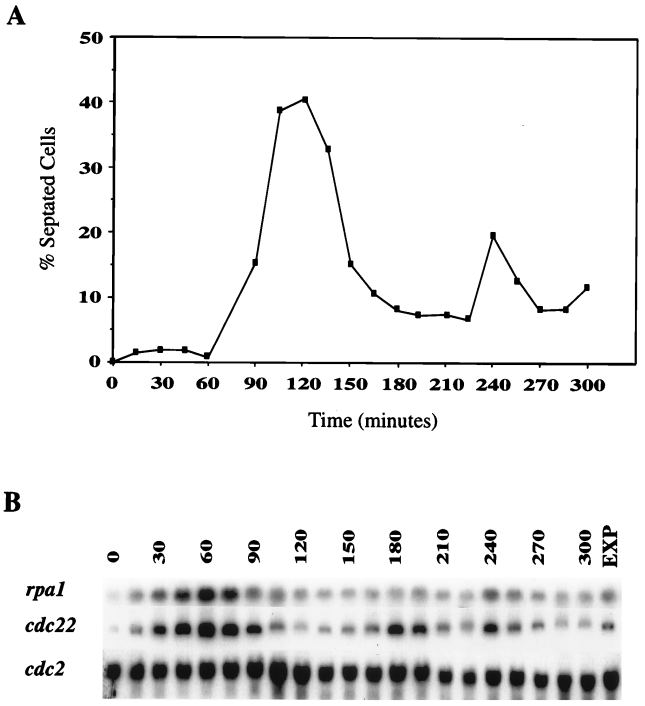

FIG. 2. Cell cycle periodicity of the *rpa1*$^+$ transcript. *cdc25-22* cells were synchronized by being shifted to the nonpermissive temperature (36°C) for 5 h and then returned to the permissive temperature (25°C). (A) The degree of synchrony was determined by scoring septation index (percent septated cells). (B) Northern blot analysis of the transcript levels for *rpa1*$^+$ and *cdc22*$^+$ from the synchronous culture monitored for two cell cycles. The blot was also probed for *cdc2*$^+$ mRNA to verify loading equivalence. Note that the 210- and 225-min time points are underloaded.

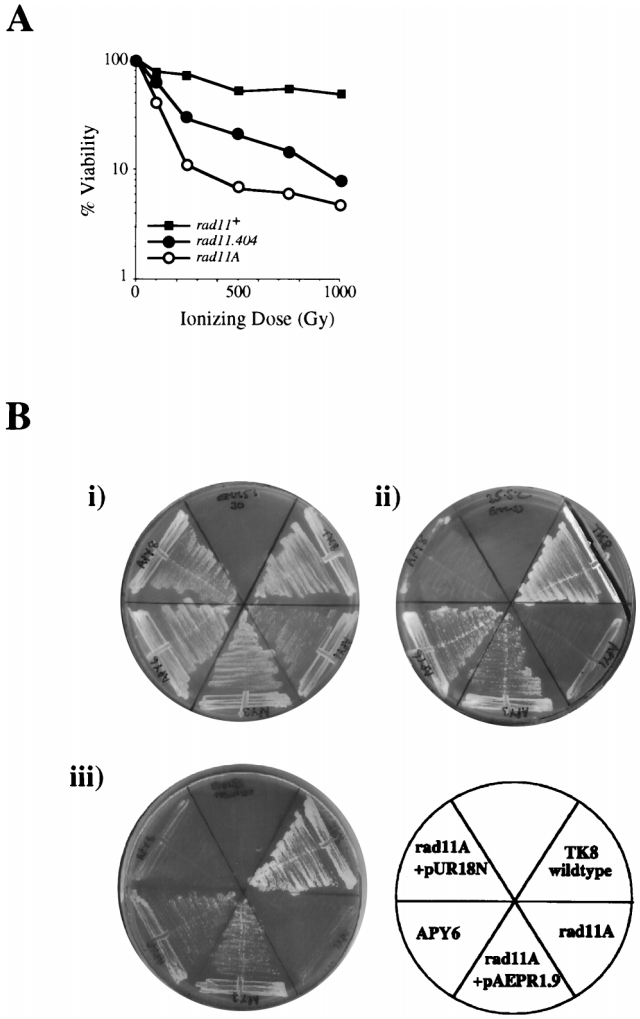

FIG. 3. Characterization of *rad11A* and complementation of its phenotypes. (A) Gamma radiation sensitivity. Cultures of equal cell density were exposed to various doses of gamma radiation, and equal volumes were plated to assess viability. (B) Rescue of *rad11A* hydroxyurea-sensitive and temperature-sensitive phenotypes by various plasmids: (i) minimal medium at 30°C, (ii) minimal medium at 36°C; and (iii) minimal medium plus 10 mM hydroxyurea at 30°C. pUR18N is a vector control, and pAEPR1.9 is the genomic clone containing the full-length *rpa1*$^+$ gene. APY6 contains a single copy of *rpa1*$^+$ integrated at the *rad11* locus.

further experiments, we observed that *rpa1*$^+$ in either multiple or single copy fully rescued the temperature sensitivity of *rad11A*. Under the same conditions, the sensitivity to hydroxyurea remained intermediate between those of mutant and wild-type strains, suggesting that the mutant phenotype was partially dominant (data not shown).

To confirm that *rpa1*$^+$ and *rad11*$^+$ are allelic, we constructed an *S. pombe* strain in which the chromosomal copy of *rpa1*$^+$ was marked with the *ura4*$^+$ gene. For this purpose, plasmid pAEPR1.9 (*rpa1*$^+$ and *ura4*$^+$) was linearized within the *rpa1*$^+$ sequence and introduced into a haploid wild-type strain to obtain a targeted integration at the *rpa1*$^+$ locus. The expected structure, consisting of two copies of the *rpa1*$^+$ gene flanking an inserted *ura4*$^+$ marker, was confirmed by Southern blot analysis. The resulting strain APY44 was crossed with the *rad11A* strain, and 24 tetrads were dissected. In all cases, the two progeny auxotrophic for uracil displayed all of the phenotypes associated with *rad11A* whereas the two uracil prototrophs were wild type in all respects (data not shown). Thus, *rad11*$^+$ and *rpa1*$^+$ are closely linked, and *rpa1*$^+$ rescues all phenotypes of *rad11A*, indicating that *rad11*$^+$ and *rpa1*$^+$ are allelic.

***rad11A* has an increased rate of meiotic recombination.** To examine the effect of *rad11A* on meiotic recombination, we determined the frequency of recombination between two closely spaced mutations in *ade6* (*ade6-M210* and *ade6-M216*) in crosses performed in the wild-type, *rad11A*, and *rpa1*$^+$ backgrounds. Meiotic recombination rates (fractions of haploid progeny prototrophic for adenine) for TK7 × TK8, APY13 × APY15, and APY43 × APY45 were 1.63×10^{-4}, 24.9×10^{-4}, and 0.91×10^{-4}, respectively. These data indicate that the rate of meiotic recombination is increased approximately 15-fold in the *rad11A* strain. Furthermore, the recombination rate in the mutant background is restored to the wild-type rate by a single copy of *rpa1*$^+$.

***rad11A* has an intact DNA damage checkpoint.** To determine whether sensitivity to irradiation was a result of a DNA repair defect or an inability to arrest mitosis following DNA damage, we examined the G_2 delay response to gamma irradiation. Cells synchronized in G_2 were exposed to increasing doses of gamma radiation and scored for passage through mitosis. When exposed to a DNA-damaging agent such as gamma radiation, wild-type cells exhibit a mitotic delay due to the DNA repair checkpoint. The length of the delay is related to the dose of radiation and therefore to the extent of the DNA damage. We observed similar mitotic delay responses for *rad11A* and wild-type cells at all gamma radiation doses examined (Fig. 5B). Thus, the radiation checkpoint is intact in *rad11A* cells and the increased sensitivity to radiation is probably an impairment of DNA repair processes and not due to a defect in arresting mitosis following DNA damage. We also examined the G_2 checkpoint following UV irradiation and found it to be intact, as *rad11A* cells again showed the same delay as wild-type cells (data not shown).

Roles of *rpa1*$^+$/*rad11*$^+$ in DNA replication and the S-phase checkpoint. To further understand the consequences of the *rad11A* mutation, we examined the temperature sensitivity and the hydroxyurea sensitivity phenotypes in more detail. The *rad11A* strain was grown to a density of 2×10^6 cells/ml at the permissive temperature of 25°C and then shifted to 36°C. Every 2 h, the number of viable cells was determined by a plating assay and cells were also fixed for flow cytometric analysis. The wild-type cells showed no change in morphology or DNA content and continued to grow exponentially for the 12 h of the experiment (Fig. 6). The *rad11A* strain maintained viability for the first 2 h after the temperature shift. By 4 h, the viability of

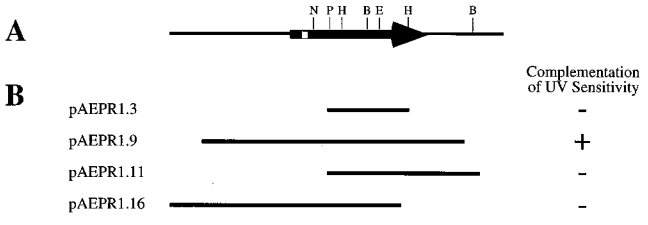

FIG. 4. Chromosomal organization of *rpa1*$^+$ and complementation of UV sensitivity. (A) *rpa1*$^+$ is located on chromosome II adjacent to the *mik1*$^+$ gene. N, *Nhe*I; P, *Pst*I; H, *Hin*dIII; B, *Bst*XI; E, *Eco*RI. The intron is indicated by the open box. The *ura4*$^+$ gene replaced the *Hin*dIII fragment to generate a null allele of the gene. (B) Complementation of the UV sensitivity of *rad11A* by the four *rpa1*$^+$ genomic clones isolated. The horizontal lines indicate the chromosomal fragments present in each clone.

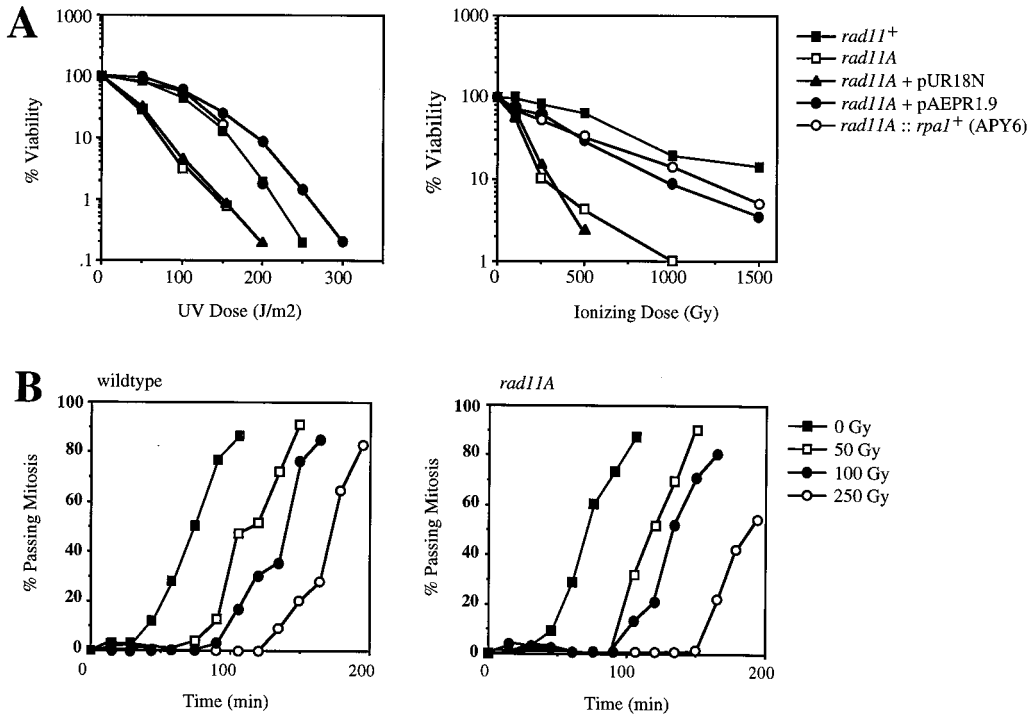

FIG. 5. Radiation sensitivity and G_2 checkpoint competence of *rad11A* cells. (A) UV and gamma radiation sensitivity. For the UV assay, equal numbers of cells were plated and exposed to various doses of UV radiation. To determine sensitivity to gamma radiation, cultures of equal cell density were exposed to various doses of gamma radiation, and equal volumes were plated to assess viability. (B) Cells were synchronized on a lactose gradient, exposed to various levels of gamma radiation, and scored for mitotic index.

the mutant cells began to decrease rapidly, reaching levels of 10% by 8 h and 4% by 12 h (Fig. 6A). Cell number continued to increase during the course of the experiment (data not shown). The observed decrease in viability of the *rad11A* cells was accompanied by a significant broadening of the flow cytometry profile, suggesting a derangement of DNA synthesis in the mutant cells (Fig. 6B). Examination of the cells by DAPI staining revealed morphological changes that correlated with the decrease in viability. At 4 h, the cells appeared relatively normal; however, by 8 h the population contained some elongated cells characteristic of cell division cycle delay (Fig. 6C). In addition, about 15% of cells at 8 h exhibited a "cut" morphology which is indicative of an abnormal mitosis/cell division in which the nuclear material either has been bisected by the septum or has segregated unequally to the two daughter cells. The number of such cut cells was significantly greater than the background of 4 to 5% observed in the *rad11A* population at the permissive temperature. By 12 h, about 30% of the cells had undergone an aberrant mitosis/cell division (Fig. 6C). These data suggest that a fraction of the cells continue to progress through the cell cycle despite abnormal DNA synthesis. The checkpoint mechanisms that restrain mitosis until normal completion of DNA replication may be compromised in the *rad11A* mutant at the nonpermissive temperature (see Discussion).

To characterize the hydroxyurea sensitivity of *rad11A* more thoroughly, we monitored the fate of a synchronous cell population incubated in the presence of the drug. Exponentially growing cultures of wild-type and *rad11A* cells were subjected to counterflow centrifugal elutriation to obtain synchronized populations of early-G_2 cells. These cells were then diluted into fresh medium containing 10 mM hydroxyurea, at a density of 2×10^6 cells/ml. Samples were removed every 2 h for determination of septation index, viability, and DNA content by flow cytometry (Fig. 7). Wild-type cells exposed to 10 mM hydroxyurea exhibit a transient G_1/S arrest due to depletion of deoxyribonucleoside triphosphates. Ultimately the cells adapt to the drug, enter an elongated S phase, and subsequently divide, with little loss in viability. In the present experiment, wild-type cells with a 1C DNA content accumulated during the first 4 h of the incubation as the synchronized G_2 population underwent a normal cell division and arrested at the G_1/S boundary (Fig. 7B). By 6 h, the wild-type cells had begun to adapt to the hydroxyurea block and had entered S phase. By 8 h, most of the cells had completed S phase and exhibited a 2C DNA content. Cell viability remained high throughout the experiment. The synchronized *rad11A* strain behaved similarly to the wild-type cells for the first several hours. In particular, the cells divided normally and arrested with a 1C DNA content (Fig. 7B). Microscopic examination of mutant or wild-type cells stained with DAPI demonstrated the presence of elongated cells with a single nucleus, consistent with a cell division cycle delay (Fig. 7C). Thus, the *rpa1*$^+$/*rad11*$^+$ gene does not appear to be required for the initial cell cycle arrest mediated by the S-phase checkpoint. However, 6 to 8 h after exposure to hydroxyurea, the fraction of viable cells in the mutant cell population decreased dramatically, to approximately 25% indicating that the majority of cells had undergone a lethal event (Fig. 7A). Cell viability continued to decline over the next 4 h, and by 12 h less than 10% of the starting cells remained viable. At 6 h, the cells had a DNA content intermediate between 1C and 2C (Fig. 7B), indicating that the decrease in viability coincided with reentry into S phase. Beginning at 8 h, an increasing fraction of mutant cells underwent aberrant mitosis/cell division (Fig. 7C). The most common phenotype was missegregation of the nuclear material such that one daughter cell

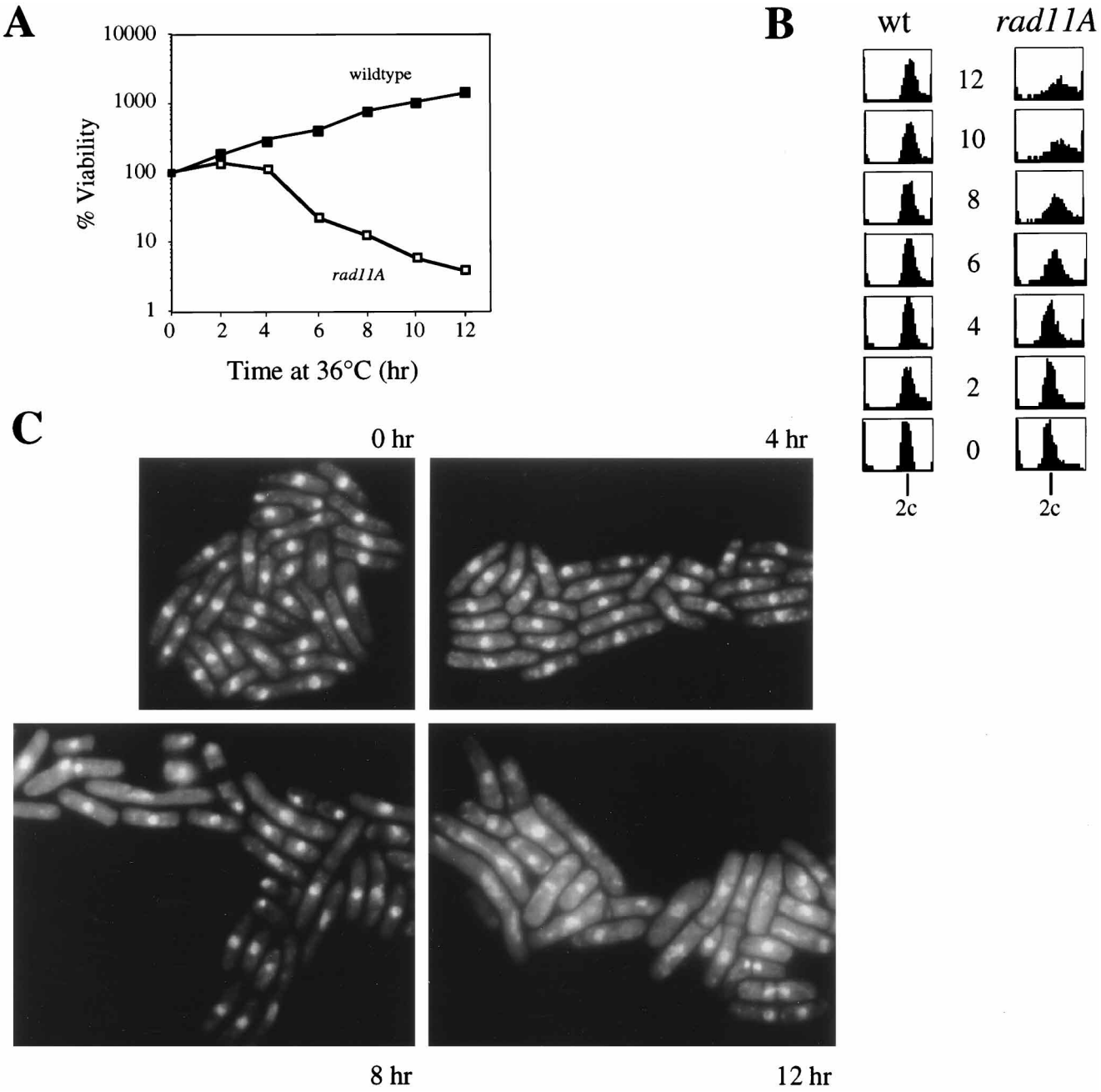

FIG. 6. *rad11A* phenotypes at the nonpermissive temperature. Cells were grown to a density of 2×10^6 cells/ml and then shifted to the nonpermissive temperature of 36°C. (A) Plating assay to determine the number of viable cells from an equal volume of culture (■, *rad11*$^+$; □, *rad11A*). (B) Flow cytometric analysis of samples taken every 2 h after the temperature shift. wt, wild type. (C) DAPI staining of *rad11A* and wild-type cells at 0, 4, 8, and 12 h after addition of hydroxyurea.

received all of the DAPI-staining material and the other daughter cell was anucleate. Cells with a classic cut phenotype and multiply septated cells were also observed in significant numbers. The overall frequencies of abnormal mitosis/cell division were 11% at 8 h and 33% at 12 h. The corresponding values for the control wild-type cells were 1 and 4%, respectively. We also observed a broadening of the FACS profile of the *rad11A* mutant at 8, 10, and 12 h. This result is consistent with aberrant DNA synthesis in the cells during growth in the presence of hydroxyurea. These data indicate that although *rad11A* arrests normally when DNA replication is inhibited by hydroxyurea, the mutant cells are unable to maintain viability during recovery from the block, presumably because abnormal DNA replication leads to irreversible genomic damage. In spite of the inability to complete a normal round of DNA replication, a significant fraction of the cells subsequently enter an aberrant mitosis/cell division typical of checkpoint-deficient cells.

DISCUSSION

To begin to address the in vivo role of RPA in DNA metabolism, we have cloned and sequenced the fission yeast gene encoding the large subunit of replication protein A, designated

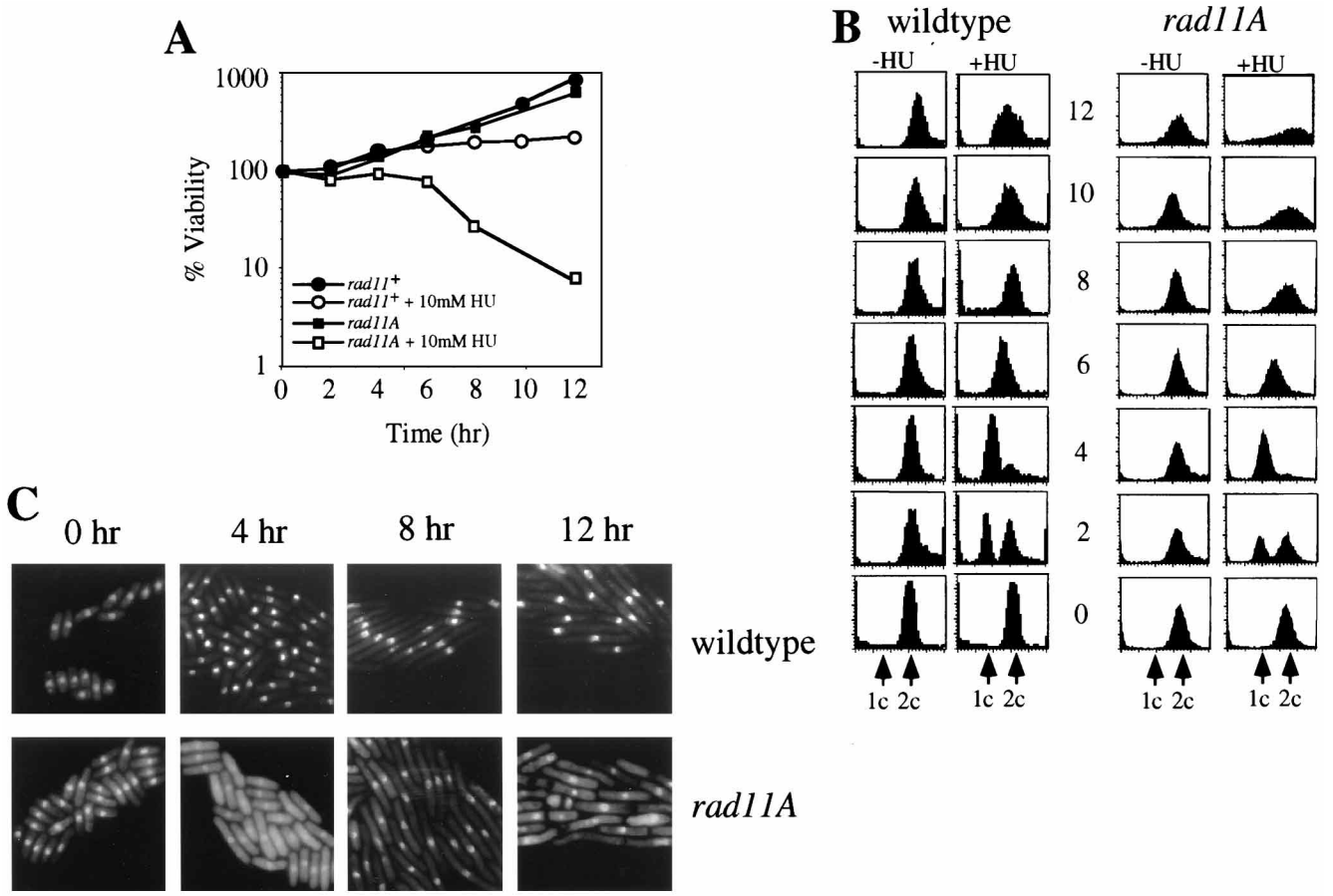

FIG. 7. Analysis of hydroxyurea sensitivity phenotype of *rad11A* at the permissive temperature. Cells were subjected to counterflow centrifugal elutriation to yield a synchronous population of early-G_2 cells, which were then returned to culture in the presence or absence of 10 mM hydroxyurea (HU). (A) Plating assay to determine the number of viable cells. (B) FACS analysis of samples taken every 2 h following synchronization. (C) DAPI staining of *rad11A* and wild-type cells at time 0, 4, 8, and 12 h after the addition of hydroxyurea.

rpa1$^+$. The predicted protein comprises 609 amino acids and is 38% identical to the human Rpa1 sequence (17). The *rpa1*$^+$ gene is unique in the fission yeast genome and is essential for viability, as demonstrated by gene disruption. This finding is in agreement with the observation that the genes encoding all three subunits of budding yeast RPA are essential (5). Analysis of mRNA levels demonstrated that the *rpa1*$^+$ transcript is regulated in a cell cycle-dependent manner. *rpa1*$^+$ mRNA accumulates to the highest levels at the G_1/S phase transition. The expression of certain other fission yeast genes involved in DNA replication (e.g., *cdc22*$^+$, which encodes the large subunit of ribonucleotide reductase) is regulated in a similar fashion during the cell cycle. Such genes are subject to the control of the start gene, *cdc10*$^+$, which encodes a transcription factor whose activity is maximal at the G_1/S transition (31). It is likely that expression of *rpa1*$^+$ is dependent upon *cdc10*$^+$.

Physical mapping experiments demonstrated that the *rpa1*$^+$ gene resides on chromosome II adjacent to the *mik1*$^+$ gene. These studies also indicated that *rpa1*$^+$ is in the vicinity of the *rad11* locus (24, 34), raising the possibility that the two genes are allelic. The *S. pombe rad* strains were originally identified in a screen for mutants sensitive to UV radiation and are thus presumed to be deficient in the ability to repair damaged DNA (41, 43). Our initial examination of strain *rad11.404* indicated that it harbored an extragenic suppressor. Removal of the suppressor resulted in a strain, designated *rad11A*, which is more sensitive to UV and gamma irradiation than the parent *rad11.404*. To formally establish a relationship between *rpa1*$^+$ and *rad11*$^+$, we demonstrated that the *rpa1*$^+$ gene was able to complement all of the phenotypes of *rad11A* in multiple and single copy. We have shown by genetic linkage analysis that *rpa1*$^+$ is allelic to *rad11*$^+$.

A thorough characterization of *rad11A* showed that it is temperature sensitive for growth, has an increased rate of meiotic recombination, and is hypersensitive to hydroxyurea. All these phenotypes were shown to be due to the same mutation by cosegregation studies, and all were masked by the suppressor mutation since they were not observed in the original strain *rad11.404*. Examination of the effects of the mutation upon DNA repair indicated that *rad11A* has an intact G_2 checkpoint when exposed to UV or ionizing radiation. Thus, the observed sensitivity of *rad11A* to radiation is likely due to defects in the repair process itself.

Analysis of the hydroxyurea sensitivity of *rad11A* in a synchronized cell population suggested that the mutant is unable to complete normal DNA synthesis under conditions of reduced nucleotide levels. When exposed to hydroxyurea, wild-type *S. pombe* cells normally arrest in the cell cycle with a 1C DNA content as the replication checkpoint is triggered. Approximately 6 h after the initial arrest, the cells recover from the hydroxyurea block and begin to synthesize DNA at a low rate. The mechanism of the recovery process is not under-

stood. *rad11A* cells exhibit the same pattern of arrest and recovery but rapidly lose viability with the onset of DNA synthesis. A significant fraction of the cells subsequently undergo an abnormal mitosis/cell division similar to that seen in *S. pombe* cells with defects in the replication checkpoint. It seems likely that the DNA synthesis that occurs in *rad11A* cells during recovery from the hydroxyurea block is abnormal or incomplete, resulting in irreversible damage to the genome and cell death. In spite of the inability to carry out normal DNA replication under these conditions, many of the mutant cells fail to respond to this situation by delaying cell division. It should be noted in this context that previous studies have shown that recovery from hydroxyurea block is also dependent on the checkpoint genes *rad17* and *hus1* (9, 16).

The phenotype of the *rad11A* mutant is similar in a number of respects to that observed with a deletion of the *cds1*$^+$ gene. *cds1*$^+$ was originally identified as a high-copy-number suppressor of a mutation in the gene encoding DNA polymerase α (35). Cells lacking *cds1*$^+$ function are hypersensitive to hydroxyurea. Like *rad11A* cells, *cds1*$^-$ cells arrest normally in hydroxyurea but exhibit a dramatic increase in cut cells when DNA synthesis resumes during recovery from the hydroxyurea block. *cds1*$^-$ cells are not sensitive to UV, suggesting that the failure to recover from hydroxyurea arrest is not due to a defect in DNA repair or failure to invoke the DNA damage checkpoint (39). Thus, both *rad11A* and *cds1*$^-$ cells are capable of establishing the replication checkpoint in response to hydroxyurea, but they appear to be defective in restraining mitosis/cell division in the face of lethal damage sustained during subsequent DNA synthesis. It is interesting that RPA appears to interact with DNA polymerase α during DNA replication in human cells (11, 14, 35). Given the genetic interactions of *cds1*$^+$ with the same polymerase, it is possible that all three proteins are part of the replication machinery during active DNA synthesis.

One simple model that could account for all of our observations is that RPA is required for the integrity of replication complexes during ongoing DNA synthesis. When mutant cells are incubated with hydroxyurea, replication complexes presumably form normally, but replication fork progression is blocked due to lack of deoxyribonucleoside triphosphates. Under these conditions replication complexes containing mutant RPA may be more labile than those containing the wild-type protein. The gradual breakdown of such complexes in *rad11A* cells may lead to defective or incomplete DNA synthesis when the cells begin to recover from the hydroxyurea block. The resulting damage to the genome causes the observed loss of viability. The apparent checkpoint defect of *rad11A* could be explained if intact replication complexes are necessary to generate the signal recognized by the checkpoint mechanism (26, 28). In this scenario, the replication complex may be intimately associated with the detection system that engages the checkpoint, and thus the checkpoint is maintained only while replication complexes remain associated with the DNA. The breakdown of replication complexes in *rad11A* cells arrested in hydroxyurea would lead to loss of the appropriate checkpoint signal and result in the cells entering an aberrant mitosis. A similar model could explain the loss of viability and checkpoint defect observed when the *rad11A* mutant is shifted to the restrictive temperature.

If this model is correct, mutations affecting any of the proteins that make up the active replication complex could in principle give rise to apparent defects in the replication checkpoint by reducing the stability of the complex when DNA synthesis is perturbed either by hydroxyurea or DNA damage. As indicated above, this hypothesis may explain the phenotype of *cds1* mutants, since the *cds1*$^+$ gene product may be part of the replication complex. The model is further supported by the observation that deletion of the *S. pombe* gene encoding DNA polymerase α can uncouple DNA replication and mitosis, giving rise to the cut phenotype (15, 20). An alternative view is that RPA may play a more direct role in the checkpoint mechanism quite apart from its role in maintaining the integrity of replication complexes. For example, it has previously been suggested that phosphorylation of the middle subunit of RPA may act as a signal for ongoing replication or repair (8, 10, 29).

The results presented in this report have demonstrated an in vivo role for RPA in DNA replication, repair, and recombination supporting the previous in vitro studies (12, 18, 22, 50, 52) and studies in budding yeast (19, 30, 47). We have also presented evidence for the involvement of RPA in an S-phase checkpoint, possibly through its role as a component of the replication complex.

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The first two authors contributed equally to this work.

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