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rqh1$^+$, a fission yeast gene related to the Bloom's and Werner's syndrome genes, is required for reversible S phase arrest

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In eukaryotic cells, S phase can be reversibly arrested by drugs that inhibit DNA synthesis or DNA damage. Here we show that recovery from such treatments is under genetic control and is defective in fission yeast *rqh1* mutants. *rqh1*$^+$, previously known as *hus2*$^+$, encodes a putative DNA helicase related to the *Escherichia coli* RecQ helicase, with particular homology to the gene products of the human *BLM* and *WRN* genes and the *Saccharomyces cerevisiae SGS1* gene. *BLM* and *WRN* are mutated in patients with Bloom's syndrome and Werner's syndrome respectively. Both syndromes are associated with genomic instability and cancer susceptibility. We show that, like *BLM* and *SGS1*, *rqh1*$^+$ is required to prevent recombination and that in fission yeast suppression of inappropriate recombination is essential for reversible S phase arrest.

Keywords: cell cycle/*hus2*/recombination/RecQ DNA helicase/*Schizosaccharomyces pombe*

Introduction

Multiple cellular mechanisms ensure the accurate transmission of genetic material from one generation to the next. These include enzymes which repair specific DNA lesions after DNA damage, as well as regulators that coordinate DNA repair and cell cycle progression. Mutations abolishing many of these processes result in genomic instability and cause cancer-prone syndromes in humans, including xeroderma pigmentosum (excision repair), colorectal cancer (mismatch repair) and ataxia telangiectasia (checkpoint control) (see Bootsma *et al*., 1995; Chung and Rustgi, 1995; Enoch and Norbury, 1995; Lehmann and Carr, 1995; Chu and Mayne, 1996). Chromosomes are likely to be particularly vulnerable to DNA damage during replication, as DNA is decondensed and partially single stranded and therefore highly accessible to damaging agents. DNA replication is, therefore, subject to exquisite regulation which ensures its coordination with DNA repair and other cell cycle processes. Control of DNA replication initiation has been studied extensively and a picture of the molecular mechanisms involved is beginning to emerge (see Peeper *et al*., 1994; Chevalier and Blow, 1996).

Replication elongation is also likely to be highly regulated, although much less is known about these controls. The presence of DNA damage or limiting concentrations of nucleotides in the cell are known to block DNA elongation (Vassilev and Russev, 1984; Friedberg *et al*., 1995). Although arrest of replication elongation could be due to mechanical or chemical blocks to DNA polymerization, it is equally possible that the pausing and resumption of DNA replication are regulated by external controls, like many other cell cycle processes (Hartwell and Weinert, 1989; Murray, 1992). Indeed, elegant studies in the budding yeast *Saccharomyces cerevisiae* have demonstrated that the slowing of DNA replication in response to DNA damaging agents requires gene products previously shown to be involved in other cell cycle checkpoints (Paulovich and Hartwell, 1995; Longhese *et al*., 1996). Here we demonstrate that reversible S phase arrest also requires protective functions that are distinct from cell cycle checkpoint controls.

We have previously studied S phase arrest in the fission yeast *Schizosaccharomyces pombe*, by isolating mutants that are sensitive to hydroxyurea (HU), a drug that blocks DNA replication by depleting deoxynucleotides. Our screening strategy was based on the observation that checkpoint-defective cells undergo an aberrant mitosis ('cut') when treated with HU (Enoch and Nurse, 1990). Under the same conditions normal cells cease DNA synthesis and arrest cell division, displaying an elongated cell morphology. By screening for mutants that 'cut' in HU, a number of checkpoint-defective HU-sensitive (*hus*) mutants were identified (Enoch *et al*., 1992). Several of the mutants were found to be allelic to previously known *rad* (radiation-sensitive) genes, including *rad3*$^+$, shown independently by Al-Khodairy and Carr to be required for checkpoint control (Al-Khodairy and Carr, 1992). *rad3*$^+$ is related to the human ataxia telangiectasia gene, *ATM* (Bentley *et al*., 1996), mutation of which causes a variety of severe symptoms, including increased rates of cancer (see Lehmann and Carr, 1995).

A single allele of *hus2*, *hus2-22* (*rqh1-h2*), was also identified in the screen for *hus* mutants (Enoch *et al*., 1992). For reasons that will become clear below, we have renamed this gene *rqh1*$^+$. Like the other *hus* mutants, *rqh1*$^-$ cells undergo an aberrant 'cut'-like mitosis in HU and are also radiation sensitive. Here we show that, unlike checkpoint mutants, *rqh1*$^-$ cells arrest DNA replication and cell division normally in response to HU but then display significant defects in chromosome segregation in the subsequent mitosis. We propose that *rqh1*$^+$ is required for recovery from S phase arrest. Our studies also indicate that *rqh1*$^+$ is required for recovery from cell cycle arrest induced by DNA damage.

We have sequenced *rqh1*$^+$ and found that it encodes a putative DNA helicase related to the products of the

human *BLM* (Bloom's syndrome) and *WRN* (Werner's syndrome) genes (Ellis *et al*., 1995; Yu *et al*., 1996) and to the *S.cerevisiae SGS1* gene (Gangloff *et al*., 1994; Watt *et al*., 1995). Bloom's and Werner's syndromes are rare congenital diseases associated with genomic instability and significant cancer predisposition (German, 1993; Yu *et al*., 1996). Sgs1 has been identified as a protein that interacts genetically and physically with type I and type II topoisomerases (Gangloff *et al*., 1994; Watt *et al*., 1995; Lu *et al*., 1996). The *rqh1*$^+$, *BLM*, *WRN* and *SGS1* genes encode proteins of similar sequence and length and contain a central domain closely related to the DNA helicase domain of the *Escherichia coli RECQ* gene product.

Increased genomic instability in *BLM*$^-$ cells is caused at least in part by elevated levels of genetic exchange, particularly between sister chromatids. Exchanges between sister chromatids and homologs can be readily detected cytologically in *BLM*$^-$ cells and such exchanges are further stimulated by exposure to DNA damaging agents (Krepinsky *et al*., 1979, 1982; Heartlein *et al*., 1987; Kurihara *et al*., 1987). Recombination is also elevated in *sgs1*$^-$ cells (Gangloff *et al*., 1994; Watt *et al*., 1996). Here we show that *rqh1*$^+$ is also required to prevent recombination, particularly during S phase arrest. Thus, the function of this subfamily of DNA helicases in regulating genetic exchange and maintaining genomic stability has been highly conserved during evolution. Moreover, prevention of inappropriate recombination is required to ensure that cell cycle progression can resume normally if S phase has been interrupted.

Results

rqh1$^+$ *encodes a RecQ-like DNA helicase*

The *rqh1*$^+$ gene, previously called *hus2*$^+$, was cloned from a genomic library by complementation of the UV sensitivity of the *rqh1-h2* (*hus2-22*) mutant (see Materials and methods). Two overlapping complementing clones were isolated, both containing the same open reading frame. The sequence of the open reading frame predicts *rqh1*$^+$ to encode a large, 1328 amino acid protein containing a 325 amino acid domain that shows sequence homology with the helicase domains of RecQ-like DNA helicases. This sequence was subsequently reported by the Sanger Center *S.pombe* genome sequencing project, accession No. Q09811. Our sequence, which agrees with that from the Sanger Center, has DDBJ/EMBL/GenBank accession No. Y09426. Related proteins are the human Blm, Wrn and RecQL proteins, *S.cerevisiae* Sgs1 and *E.coli* RecQ (Figure 1A). Figure 1B shows a sequence alignment of the helicase domains of the members of the RecQ-like family of helicases, including Rqh1. The Rqh1 helicase domain shows most similarity to the Blm helicase domain (55% amino acid identity) and least similarity to the Wrn helicase domain (40% amino acid identity). While all six members of the RecQ helicase family share sequence homology within the core helicase domain, Rqh1 appears to belong to a subfamily consisting of Rqh1, Blm, Wrn and Sgs1. These four proteins are approximately the same length, considerably longer than the *E.coli* prototype. They also show an extended region of sequence homology and have a similar positioning of the helicase domain (see Figure 1A). In addition, although the N- and C-termini of Rqh1, Blm, Wrn and Sgs1 show little sequence similarity, they are all rich in charged and polar amino acids, especially serines, and have patches of acidic residues (Figure 1A) (Ellis *et al*., 1995; Rothstein and Gangloff, 1995; Yu *et al*., 1996).

Analysis of the rqh1 deletion strain

The *rqh1* null phenotype was determined by disruption of the *rqh1*$^+$ gene in the *S.pombe* genome. Most of the *rqh1*$^+$ open reading frame, including the entire N-terminus, all of the region encoding the helicase domain and half of the C-terminus, was replaced with the *ura4*$^+$ gene (see Figure 2A and Materials and methods). The strain carrying the *rqh1* deletion (*rqh1*Δ) is viable, although in rich medium, cultures of *rqh1*Δ cells, like those of *rqh1-h2*, have a 38% longer doubling time than wild-type cells (data not shown; Enoch *et al*., 1992). *rqh1*Δ cells show no other obvious defects under normal growth conditions. Appropriate crosses established that *rqh1*Δ and *rqh1-h2* are allelic.

We have previously shown that the *rqh1-h2* mutant is sensitive to the DNA replication inhibitor hydroxyurea (HU) and to UV irradiation (Enoch *et al*., 1992). The HU sensitivities of *rqh1*Δ cells (TE767, see Table 1) and *rqh1-h2* cells (TE232, see Table 1) were compared. During a 10.5 h incubation in 10 mM HU, wild-type cells remained largely viable, but the viability of *rqh1*Δ and *rqh1-h2* cells dropped >50-fold (Figure 2B). The sensitivities of *rqh1*Δ, *rqh1-h2* and wild-type cells to UV irradiation were also examined. Unlike wild-type cells, both *rqh1*Δ and *rqh1-h2* cells were markedly sensitive to low doses of UV irradiation, with the viability of *rqh1*$^-$ cells dropping >500-fold upon irradiation at 200 J/m^2 (Figure 2C). Since *rqh1*Δ and *rqh1-h2* cells behaved identically in these assays, we believe that *rqh1-h2* is a null allele.

rqh1Δ cells undergo aberrant mitoses upon treatment with HU or UV irradiation

rqh1-h2 mutants were initially identified in a screen for checkpoint mutants that were unable to arrest the cell cycle in response to HU. Like known checkpoint mutants, *rqh1* mutants are hypersensitive to HU and HU-treated *rqh1-h2* cells undergo septation in the absence of chromosome segregation (Enoch *et al*., 1992). This results in cells where the septum has either bisected the single nucleus or divided the cell such that one daughter is anucleate. We refer to this as the 'cut' phenotype, as it resembles the phenotype of previously described *cut* mutants, which have defects in chromosome segregation (Hirano *et al*., 1986). In contrast, wild-type cells arrest the cell cycle when they are incubated in HU and become elongated, because they continue to grow without dividing.

*rqh1*Δ cells, like *rqh1-h2* mutants, show the 'cut' phenotype when incubated in HU, while wild-type cells do not (compare Figure 3A and B). The 'cut' cells are first observed after 7 h incubation in HU (see Figure 3E) and are noticeably elongated, indicating that the aberrant mitosis occurs after a cell cycle delay (the cells in Figure 3A and B had been incubated in HU for 9 h). This is in contrast to checkpoint mutants, which do not elongate at all in HU because cell cycle progression is not delayed. Cells with the 'cut' phenotype also accumulate much more rapidly in cultures of checkpoint mutants than they do in

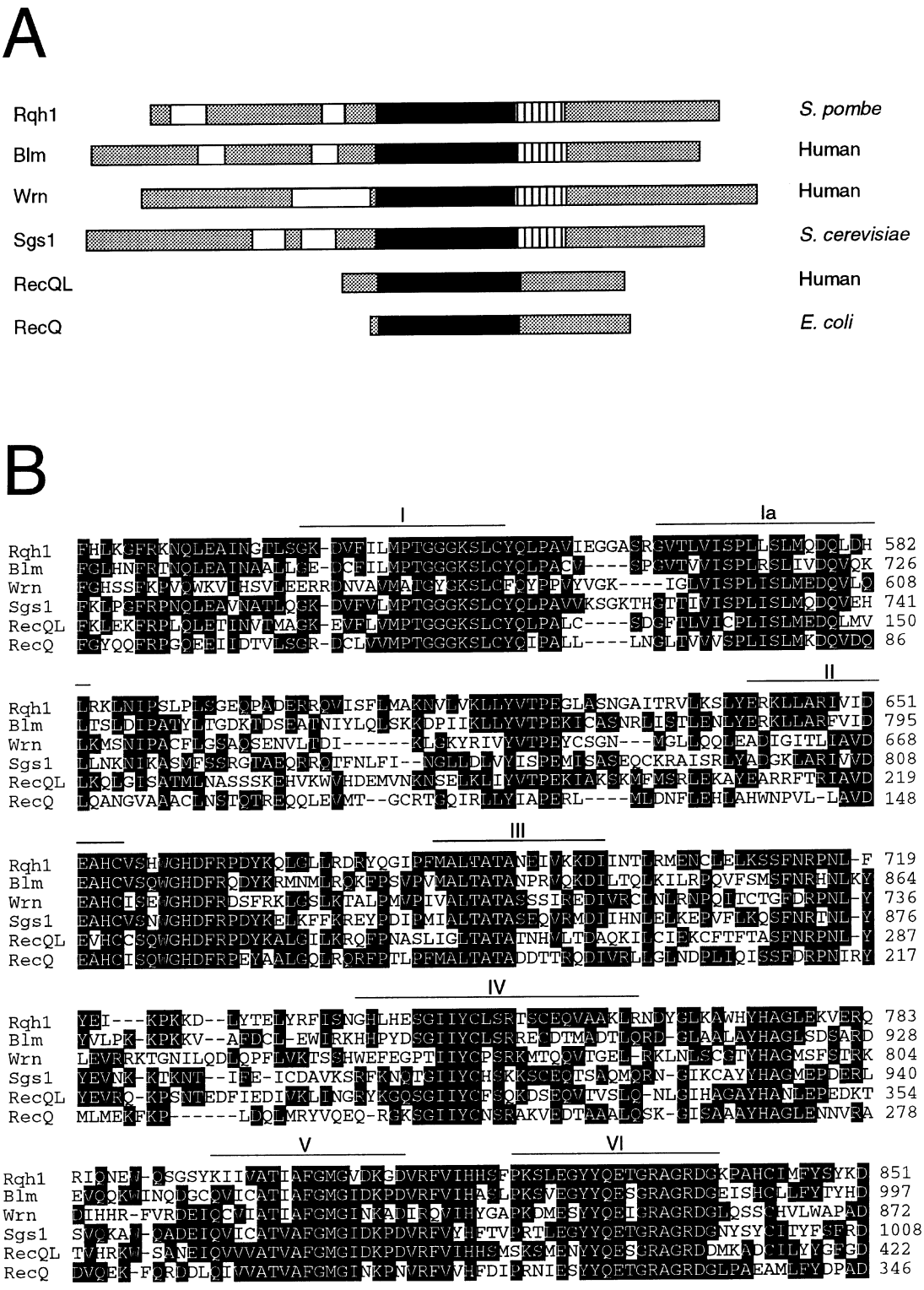

Fig. 1. *rqh1*$^+$ encodes a RecQ-like DNA helicase. (**A**) Schematic representation of the members of the RecQ-like DNA helicase family; the name of the gene product is shown on the left and the organism is on the right. The conserved helicase domains are shown as black boxes; regions of extended homology are shown as striped boxes; acidic domains are shown as white boxes; regions with no sequence homology are stippled. The proteins were arranged by aligning the helicase domains. (**B**) Sequence alignment of the helicase domains of the members of the RecQ-like DNA helicase family (black boxes in A). The gene product names are shown on the left, the amino acid position for each protein on the right. The sequence alignment was carried out using the DNASTAR Megalign program. Residues that are identical to the consensus are shown in white on a black background; dashes indicate spaces introduced by the program to maintain sequence alignment. The positions of the seven helicase domains (Gorbalenya *et al.*, 1989) are indicated by lines above the sequence. The *rqh1*$^+$ sequence has been submitted to the DDBJ/EMBL/GenBank database, accession No. Y09426. This sequence has also been reported by the Sanger Center *S.pombe* genome sequencing project, accession No. Q09811.

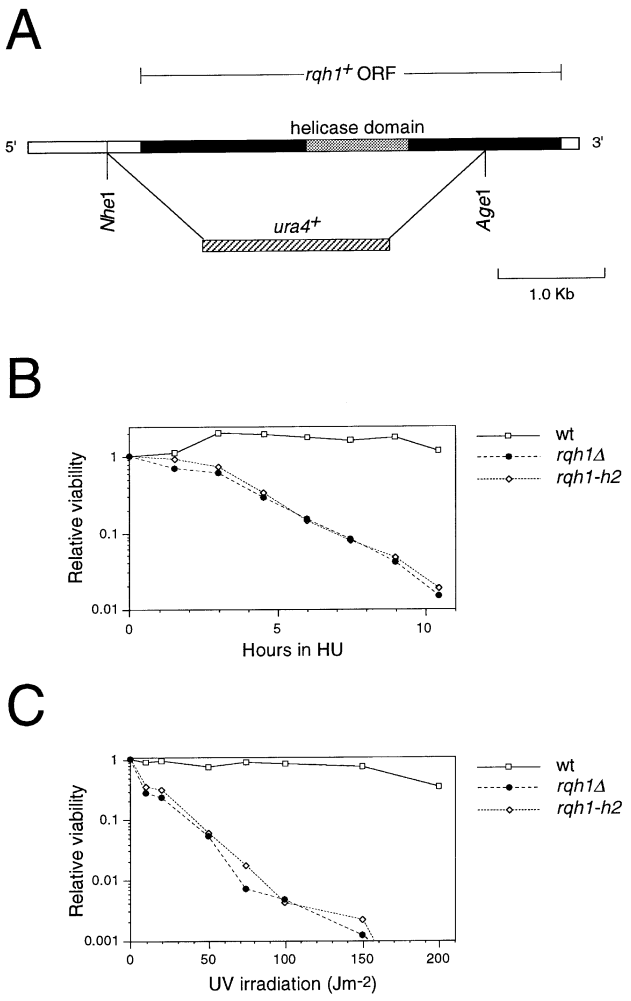

Fig. 2. Construction and analysis of the *rqh1* deletion mutant. (**A**) Diagram of the DNA construct used to make the *rqh1* deletion mutant. The open reading frame of the *rqh1*$^+$ gene is shown as a black box with a stippled box representing the region of the gene encoding the helicase domain. The *ura4*$^+$ gene is shown as a striped box. The *ura4*$^+$ gene was inserted between the *Nhe*I and *Age*I sites in the *rqh1*$^+$ gene *in vitro*. (**B**) *rqh1*Δ and *rqh1-h2* cells are sensitive to HU. The viability of wild-type (TE271, see Table I), *rqh1*Δ (TE767, see Table I) and *rqh1-h2* (TE232, see Table I) cells was assayed by colony formation at times after the addition of 10 mM HU to the cultures. The relative viability was calculated by dividing the number of viable cells at each time point by the number of viable cells before addition of HU to the culture (see Materials and methods). (**C**) *rqh1*Δ and *rqh1-h2* cells are sensitive to UV irradiation. The viability of wild-type (TE271, see Table I), *rqh1*Δ (TE767, see Table I) and *rqh1-h2* (TE232, see Table I) cells was assayed by colony formation after UV irradiation. The viability of cells after irradiation was expressed relative to the viability of unirradiated cells (see Materials and methods).

*rqh1*Δ cultures, being readily observed after only 3 h incubation in HU (Enoch *et al*., 1992).

As shown in Figure 3B, by the time the *rqh1*Δ cells cut, wild-type cells are no longer particularly elongated, suggesting that they have re-entered the cell cycle after a period of cell cycle arrest. To investigate whether this was the case, wild-type (TE271, see Table I) and *rqh1-h2* (TE232, see Table I) cells were incubated in 10 mM HU and the number of cells in the culture and the percentage of cells showing the 'cut' phenotype were analyzed at regular intervals. As Figure 3E shows, the cell number continues to increase in cultures of both wild-type and *rqh1*$^-$ cells for the first 3 h after addition of HU. This is because exponentially growing *S.pombe* cells are predominantly in the G_2 phase of the cell cycle, so after addition of HU these cells divide once before they encounter the S phase block. For the next 4 h neither wild-type nor *rqh1*$^-$ cells divide, showing that the cell cycle is blocked. After ~7 h in HU, however, the number of cells in both the wild-type and *rqh1*$^-$ cultures starts to increase once more, showing that the cells have overcome the HU-induced block and have re-entered the cell cycle. This establishes that HU delays *rqh1*$^-$ and wild-type cell cycles to the same extent. Thus, by definition, *rqh1*$^-$ cells do not have a defect in the S–M checkpoint (Hartwell and Weinert, 1989). Resumption of the cell cycle after 7 h in HU is consistent with previous studies showing that HU only blocks cell division temporarily in *S.pombe* (Sazer and Nurse, 1994). Microscopic analysis shows that *rqh1*$^-$ cells showing the 'cut' phenotype only start to accumulate as wild-type and *rqh1*$^-$ cells re-enter the cell cycle (Figure 3E). Thus *rqh1*$^-$ cells may 'cut' because they are unable to segregate chromosomes after S phase arrest.

We also investigated the morphology of *rqh1*Δ cells (TE767, see Table I) after UV irradiation (see Materials and methods). Figure 3C shows *rqh1*Δ cells 13 h after irradiation at 150 J/m^2. Like *rqh1*Δ cells treated with HU, UV-irradiated *rqh1*Δ cells 'cut'. In contrast, UV-irradiated wild-type cells and unirradiated *rqh1*Δ cells do not show the 'cut' phenotype (Figure 3D and data not shown). Like HU-treated cells, UV-irradiated *rqh1*$^-$ cells that are 'cutting' are elongated compared with irradiated wild-type cells (compare Figure 3C and D), indicating that aberrant mitosis has taken place after a cell cycle delay. Cell number measurements confirm that, as in HU-treated cells, the aberrant mitosis in UV-irradiated *rqh1*$^-$ cells does not occur until wild-type cells have recovered from UV-induced arrest and re-entered the cell cycle (data not shown). This suggests that, like *rqh1*$^-$ cells incubated in HU, UV-irradiated *rqh1*$^-$ cells may have a chromosome segregation defect but are not checkpoint defective.

'Cut' rqh1$^-$ *cells have completed DNA replication*

Previously described S–M checkpoint mutants 'cut' in HU without replicating their DNA. To investigate whether 'cut' *rqh1*$^-$ cells have completed DNA replication, we examined the DNA content and cell morphology of wild-type and *rqh1*$^-$ cells during recovery from HU arrest. Cultures of wild-type (TE271, see Table I) and *rqh1-h2* (TE232, see Table 1) cells were incubated in medium containing 10 mM HU for 4 h, then filtered into fresh medium without HU. Samples were taken from the culture at 20 min intervals after removal of HU and fixed for FACS analysis, to determine the DNA content of the cells, and examined microscopically for 'cuts' (see Materials and methods). As shown in Figure 4A, wild-type and *rqh1*$^-$ cells resume and complete DNA replication with similar kinetics after HU arrest. In both wild-type and *rqh1*$^-$ cells DNA replication starts between and 20 and 40 min after removal of HU and is complete after 80 min. Figure 4B shows that *rqh1*$^-$ cells showing the 'cut' phenotype were first observed 80 min after removal of HU and were still accumulating at 140 min, well after DNA replication was complete. Thus *rqh1*$^-$ cells are able to arrest the cell cycle upon inhibition of S phase and can

Table I. *Schizosaccharomyces pombe* strains and DNA plasmids described in this paper

Strain	Markers
TE232	*rqh1-h2 h*$^+$
TE271	972 *h*$^-$
TE480	diploid *ade-M216/ade-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 h*$^+$*/h*$^+$ (GP109)
TE558	diploid *ade6-M210/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18 h*$^-$*/h*$^-$
TE725	*ade6-L469 leu1-32 h*$^-$
TE728	*ade6-M26 his3-27 mat2-102*
TE730	*rqh1-h2 ade6-M26 his3-27 mat2-102*
TE744	*rqh1-h2 ade6-L469 leu1-32 h*$^-$
TE745	diploid *ade6-L469/ade6-M26 leu1-32/leu1*$^+$ *his3-27/his3*$^+$ *h*$^-$*/mat2-102*
TE747	diploid *rqh1-h2/rqh1-h2 ade6-L469/ade6-M26 leu1-32/leu1*$^+$ *his3-27/his3*$^+$ *h*$^-$*/mat2-102*
TE767	*rqh1::ura4*$^+$ *ura4-D18 h*$^-$
TE786	*rqh1::ura4*$^+$ *ura4-D18 ade6-M210* containing Ch^{16}
TE788	*ade6-M210* containing Ch^{16}
Plasmid	**Construct**
pTE151	rqh1B
pTE152	rqh1A
pTE436	*rqh1*$^+$ disruption construct

complete bulk DNA replication in a normal manner once HU has been removed from the culture. However, even though DNA has been replicated, normal chromosome segregation cannot take place in many of the cells. These results suggest that correct chromosome segregation after S phase arrest requires special functions that are absent in *rqh1* mutant cells.

rqh1$^+$ *is required for accurate chromosome segregation, especially after HU arrest*

The above experiments establish that *rqh1*$^-$ cells are defective in chromosome segregation after HU treatment. Similar aberrant mitoses are observed in fission yeast *top2* and *cut* mutants, which lack functions required for chromosome segregation (Uemura and Yanagida, 1984; Hirano *et al*., 1986). To analyze the chromosome segregation defect in *rqh1* mutants further, we measured the rate of loss of a non-essential minichromosome from *rqh1*$^-$ and wild-type cells.

*rqh1*Δ and wild-type strains of *S.pombe* containing the *ade6-M210* allele at the genomic *ade6* locus and a single copy of the non-essential, centromeric minichromosome Ch^{16} (Niwa *et al*., 1986) were constructed (TE786 and TE788 respectively, see Table I and Materials and methods). Since Ch^{16} contains the *ade6-M216* allele, which shows intragenic complementation with *ade6-M210* (Leupold and Gutz, 1964), cells containing the minichromosome are *ade*$^+$, but those that have lost Ch^{16} are *ade*$^-$. The chromosome loss rates of wild-type and *rqh1*$^-$ cells were calculated by determining the proportion of cells that became *ade*$^-$ in a known number of generations (see Materials and methods). As shown in Figure 5A, under normal growth conditions the rate of chromosome loss for wild-type cells was 1.2×10^{-4} per generation, in close agreement with the rate previously observed by Niwa *et al.* (1986). In contrast, *rqh1*$^-$ cells were 15 times more likely to lose Ch^{16}, with a loss rate of 1.8×10^{-3} per generation. These results suggest that *rqh1*$^-$ cells lack functions required for accurate chromosome segregation even under normal growth conditions, which may account for the longer doubling time of cultured *rqh1*$^-$ cells (see above). Chromosome loss rates were also measured in cells following a 4 h incubation in 10 mM HU (for details see Materials and methods). Under these circumstances the wild-type loss rate was elevated 4.7-fold to 5.6×10^{-4} per generation, while the rate of chromosome loss from *rqh1*$^-$ cells was elevated to a rate of 1.5×10^{-2} per generation. Thus the chromosome loss rate from HU-treated *rqh1*$^-$ cells was more than 8-fold higher than untreated *rqh1*$^-$ cells and nearly 30-fold higher than the rate of loss from HU-treated wild-type cells (Figure 5A). Indeed, minichromosome loss rates after HU treatment of *rqh1*$^-$ cells may be even higher than those figures stated here. After a 4 h incubation in HU ~65% of the *rqh1*$^-$ cells were unable to recover from the S phase arrest and many went on to form the 'cut' phenotype. Since the 'cut' phenotype could be an extreme manifestation of a chromosome segregation defect, many of the cells that did not survive the incubation in HU may have shown chromosome loss. As we can only measure loss rates for those cells that survive, our numbers may represent an underestimation for minichromosome loss after HU treatment. UV-irradiated *rqh1*$^-$ cells also display significantly elevated rates of chromosome loss (data not shown). Thus *rqh1*$^+$ function appears to be required for proper chromosome maintenance under normal growth conditions and is even more important after S phase arrest or DNA damage.

rqh1$^+$*, like BLM, negatively regulates recombination*

As described above, the *rqh1*$^+$ gene product is structurally similar to the product of the *BLM* gene. *BLM*$^-$ cells show substantially elevated levels of recombination, with a particularly striking increase in sister chromatid exchange (SCE). SCE levels in *BLM*$^-$ cells are further elevated by treatment with agents that induce DNA damage (Krepinsky *et al*., 1979, 1982; Heartlein *et al*., 1987; Kurihara *et al*., 1987). Recombination rates are also elevated in *S.cerevisiae* cells lacking the *SGS1* gene (Gangloff *et al*., 1994; Watt *et al*., 1996). To determine whether *rqh1*$^-$ cells have a similar defect, the rate of mitotic recombination between *ade6* heteroalleles was measured in homozygous *rqh1*$^-$ and wild-type diploids. Stable wild-type and *rqh1-h2*

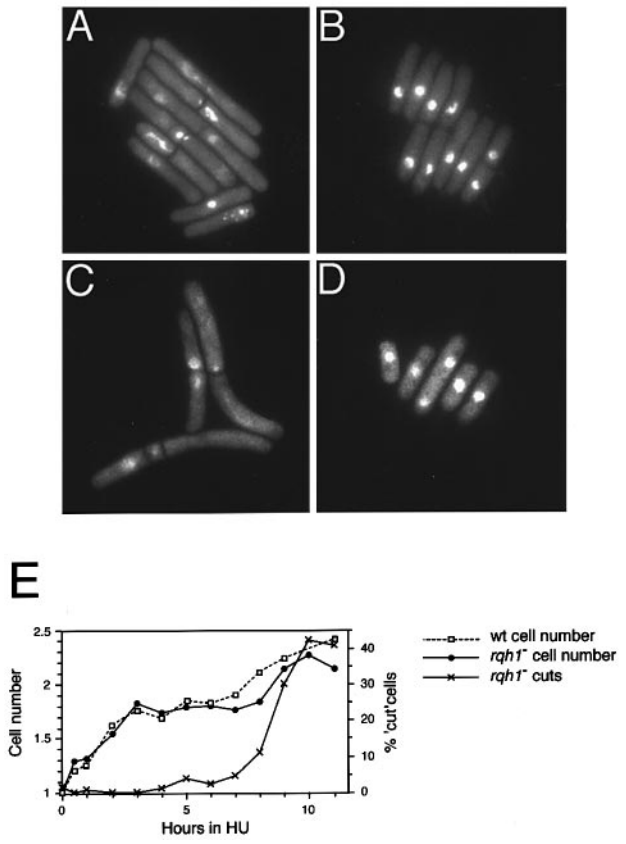

Fig. 3. HU-treated and UV-irradiated *rqh1*Δ cells 'cut' when they recover from the cell cycle block and re-enter the cell cycle. (**A**) *rqh1*Δ cells (TE767, see Table 1) and (**B**) wild-type cells (TE271, see Table 1) after a 9 h incubation in 10 mM HU. (**C**) *rqh1*Δ cells (TE767, see Table I) and (**D**) wild-type cells (TE271, see Table I) 13 h after UV irradiation at 150 J/m^2. Wild-type cells are not extended because they have resumed cell division after initially arresting in response to HU or UV (see E and text for details). All cells were heat fixed and stained with 4′6-diamidino-2-phenylindole (DAPI), which stains nuclear material brightly. The cytoplasm also stains weakly, allowing visualization of the septa as dark lines across the cells. Note that in (A) and (C) many cells show the 'cut' phenotype, with the septum either forming across the nuclear material or dividing the cell such that one daughter is anucleate. (**E**) HU-treated *rqh1-h2* cells cut only once the cells have recovered from the cell cycle block. 10 mM HU (final concentration) was added to asynchronous cultures of wild-type (TE271, see Table I) and *rqh1-h2* (TE232, see Table I) cells growing in rich medium. Samples were removed for analysis of cell number using a hemocytometer and analysis of 'cut' formation by microscopic examination of DAPI stained cells. Cell number data are shown as a proportion of the cell number when HU was added to the culture.

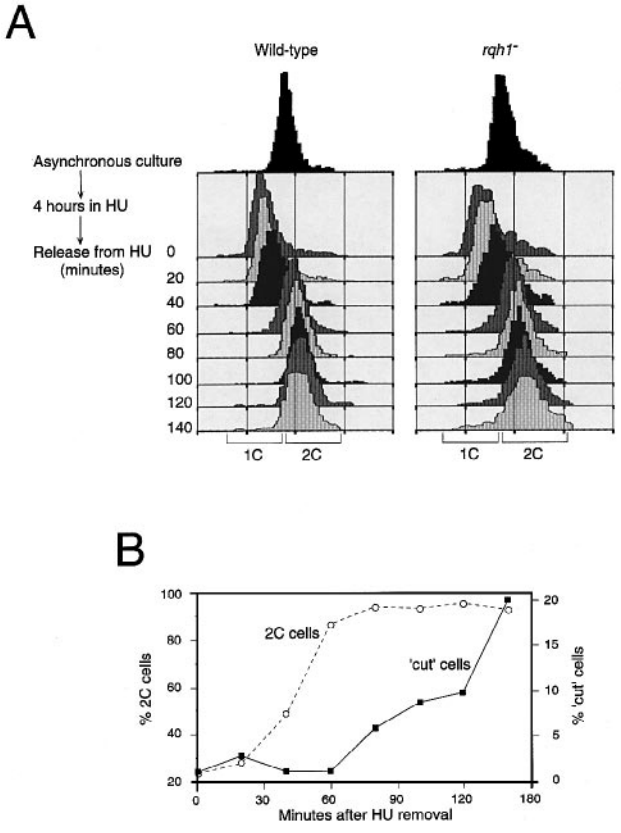

Fig. 4. *rqh1*$^-$ cells are not checkpoint defective but are deficient in recovery from S phase arrest. 10 mM HU (final concentration) was added to asynchronous cultures of wild-type (TE271, see Table I) and *rqh1-h2* (TE232, see Table I) cells growing in rich medium. After 4 h in HU the cells were filtered and transferred to rich medium without HU. Samples were removed and fixed in ethanol at 20 min intervals after removal of HU. The ethanol-fixed cells were either stained with propidium iodide for analysis of DNA content by FACS or stained with DAPI and examined microscopically. (**A**) FACS analysis of cells released from S phase arrest. Cultures of wild-type and *rqh1-h2* cells were treated with HU for 4 h then released, as described above. Note that asynchronous cultures of fission yeast have a predominantly 2C DNA content. (**B**) Comparison of the percentage of *rqh1-h2* cells showing the 'cut' phenotype and the percentage with a 2C DNA content after HU treatment. The percentage of cells showing the 'cut' phenotype was determined by microscopic examination of DAPI stained cells. The percentage of cells with a 2C DNA content was determined by FACS analysis, using the bracket indicated in (A).

diploid cells were constructed that were heterozygous at the *ade6* locus, carrying different *ade6* alleles with mutations at either end of the *ade6* gene (TE745 and TE747 respectively; see Table I and Materials and methods). Homologous recombination occurring between the hetero-alleles results in restoration of the *ade*$^+$ phenotype. The levels of recombination in these wild-type and *rqh1*$^-$ cells under normal growth conditions and after a 4 h incubation in HU were calculated by determining the proportion of cells that became *ade*$^+$ in a known number of generations (see Materials and methods).

The levels of recombination occurring under normal growth conditions were almost identical in wild-type and *rqh1*$^-$ cells, with rates of 7.50×10^{-7} and 7.65×10^{-7} per generation respectively (Figure 5B, left panel). This is comparable with rates determined in *S.cerevisiae* for similar intervals (Watt *et al*., 1996). After a 4 h incubation in 10 mM HU, recombination was stimulated in both cultures, but the effect was much more dramatic in *rqh1*$^-$ cells (Figure 5B, right panel; note that a different scale is used in each panel). While the wild-type recombination rate increased 80-fold to 6.14×10^{-5}, the *rqh1*$^-$ rate increased >800-fold to 6.36×10^{-4}. Thus *rqh1*$^+$, like *BLM* and *SGS1*, prevents recombination, particularly after S phase arrest. Like the minichromosome loss data, our figures for recombination rates after HU treatment of *rqh1*$^-$ cells may be lower than the true rates, since the cells that died upon HU treatment may have the highest levels of recombination.

Our results show that *rqh1*$^+$ is required to prevent excessive recombination after S phase arrest. High levels of recombination could cause the chromosome segregation

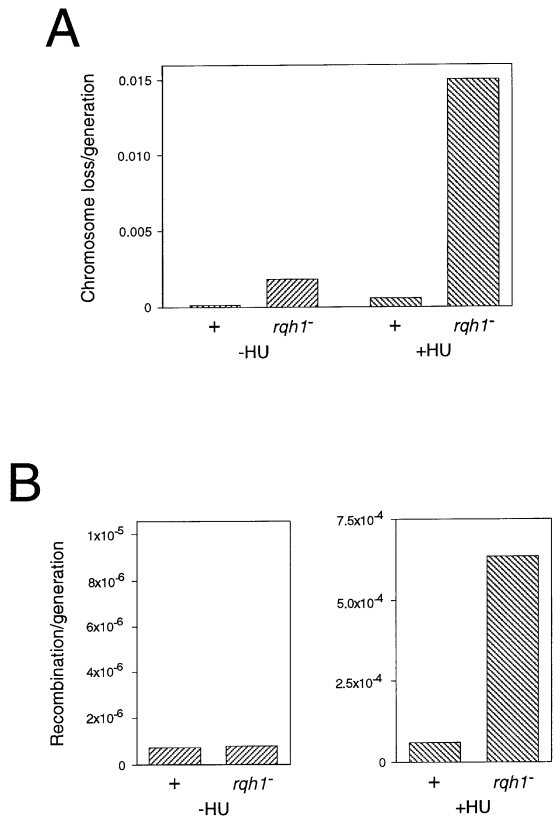

Fig. 5. Chromosome instability and recombination in HU-treated and untreated wild-type and *rqh1*$^-$ cells. (**A**) Chromosome loss. Wild-type and *rqh1*Δ strains of fission yeast were constructed containing a single copy of the non-essential minichromosome Ch^{16} (strains TE788 and TE786 respectively, see Table I). The rate of loss of Ch^{16} was calculated as described in Materials and methods. The left two columns show the rate of chromosome loss per generation from cells under normal growth conditions. The right two columns show the rate of chromosome loss per generation from cells treated with HU for 4 h. The rates of loss for wild-type and *rqh1*Δ cells under normal growth conditions were 1.2×10^{-4} and 1.8×10^{-3} respectively. After HU treatment the values were 5.6×10^{-4} and 1.5×10^{-2} respectively. (**B**) Mitotic homologous recombination. Stable homozygous wild-type or *rqh1-h2* diploids were constructed containing two *ade6* heteroalleles (strains TE745 and TE747 respectively, see Table I). Restoration of an *ade*$^+$ phenotype to these diploids occurred when homologous recombination occurred between the mutations. The rate of recombination was calculated as described in Materials and methods. The left panel shows the rate of recombination per generation in wild-type and *rqh1*$^-$ cells under normal growth conditions. The right panel shows the rate of recombination per generation in wild-type and *rqh1*$^-$ cells incubated in 10 mM HU. Note the differences in scales between the two panels. The rates of recombination for wild-type and *rqh1*$^-$ cells under normal conditions were 7.50×10^{-7} and 7.65×10^{-7} respectively. After HU treatment the values were 6.14×10^{-5} and 6.36×10^{-4} respectively.

defect in HU-treated *rqh1*$^-$ cells, as it may be impossible to segregate sister chromatids that are linked via recombination intermediates.

Discussion

rqh1$^-$ *cells are defective in recovery from S phase arrest*

The *rqh1-h2* mutant was first identified in a screen for HU-sensitive checkpoint mutants. Here we describe characterization of the *rqh1* deletion strain and the *rqh1-h2* mutant, which appears to be a null allele (Figure 2). Like checkpoint mutants, *rqh1*$^-$ cells are hypersensitive to HU and *rqh1*$^-$ cells incubated in HU display the 'cut' phenotype (Figures 2 and 3). However, unlike checkpoint mutants, *rqh1*$^-$ cells arrest cell division in response to HU. Moreover, when released from the S phase arrest, *rqh1*$^-$ cells complete bulk DNA replication in an apparently normal fashion (Figure 4). Nevertheless, *rqh1*$^-$ cells are unable to segregate their chromosomes in the mitosis after S phase arrest, leading to the 'cut' phenotype. These experiments show that *rqh1* mutants are not checkpoint defective, but rather are unable to recover from S phase arrest. Thus reversible S phase arrest entails more than stopping and resuming DNA synthesis. Additional processes, which are dependent on *rqh1*$^+$, are required to ensure normal segregation of chromosomes in the mitosis that follows arrest of DNA replication. *rqh1*$^+$ may be required either while DNA replication is arrested or after it resumes, to ensure that the arrest can be reversed.

Under normal growth conditions *rqh1*$^-$ mutants have a modestly elevated generation time and show enhanced rates of minichromosome loss. Although we were not able to observe elevated recombination levels in untreated *rqh1*$^-$ cells, we have only analyzed recombination between one pair of heteroalleles and only over a small interval and thus our assay may not have been sensitive enough to detect increased recombination. It is possible that elevated recombination levels might be revealed if larger intervals were examined. Thus we believe that *rqh1*$^+$ could have a function during the normal cell cycle which becomes essential after S phase arrest or DNA damage. Alternatively, *rqh1*$^+$ might play a different role during the normal cell cycle, unrelated to its function after S phase arrest or DNA damage.

Genetic control of S phase arrest and recovery

Analysis of the fission yeast *checkpoint* genes has previously shown that normal S phase arrest requires at least two genetically controlled processes; arrest of mitosis by negative regulation of the cyclin dependent kinase Cdc2 and induction of functions that are necessary to allow recovery of cells after S phase arrest (Enoch *et al*., 1992; Al-Khodairy *et al*., 1994). Both recovery and checkpoint control are abolished by mutations in *checkpoint rad* genes (*hus1*$^+$, *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$ and *rad26*$^+$). However, mutations in cell cycle control genes (*cdc2*$^+$, *cdc25*$^+$, *weel*$^+$ and *mik1*$^+$) only abolish the checkpoint function (Enoch and Nurse, 1990; Enoch *et al*., 1991, 1992; Sheldrick and Carr, 1993). Here we show that recovery, but not checkpoint control, is abolished by mutation of the *rqh1*$^+$ gene. A possible model linking checkpoint control and recovery is presented in Figure 6. The *checkpoint rad* gene products are proposed to function early during S phase arrest, possibly generating a signal that engages the cell cycle checkpoint and activates *rqh1*$^+$-dependent recovery processes. Thus checkpoint control and recovery are both dependent on *checkpoint rad* gene function, although they are independent processes. Interactions between the *checkpoint rad* gene products and downstream gene products could be direct or indirect.

rqh1$^-$ cells are also sensitive to UV irradiation. Again this is not due to a checkpoint defect, as *rqh1*$^-$ cells arrest upon UV irradiation and only 'cut' when they re-enter the cell cycle and try to segregate their chromosomes.

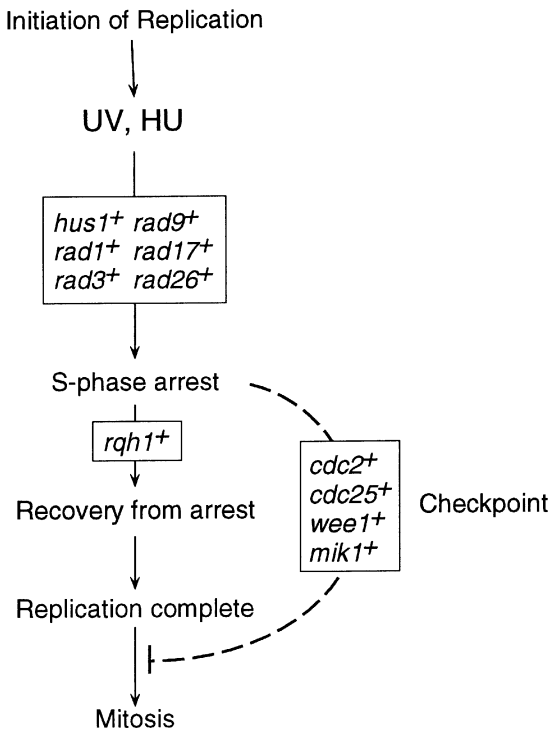

Fig. 6. Genetic control of reversible S phase arrest. When DNA replication is inhibited at the elongation stage by HU, many genes are required to ensure that inhibition and subsequent recovery from S phase arrest occur with minimal loss of cellular viability. The *checkpoint rad* genes (*hus1*$^+$, *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$ and *rad26*$^+$) are required both for the checkpoint that prevents mitosis occurring in the absence of completed DNA replication (dashed line) and for functions that allow recovery from S phase arrest. The cell cycle control genes (*cdc2*$^+$, *cdc25*$^+$, *weel*$^+$ and *mik1*$^+$) are only required for the checkpoint (dashed line). *rqh1*$^+$ is required for functions that allow the recovery of cells from S phase arrest.

Possibly this UV sensitivity is also due to a defect in S phase recovery, as UV-induced lesions could be inhibiting ongoing DNA replication. Previous studies have shown that mutants lacking S phase recovery functions are particularly sensitive to UV during S phase (Al-Khodairy *et al*., 1994).

It is remarkable that arrest and recovery in response to HU or UV irradiation in fission yeast requires two genes, *rad3*$^+$ and *rqh1*$^+$, related to human genes mutated in two different cancer-prone syndromes, ataxia telangiectasia and Bloom's syndrome. This suggests that reversible S phase arrest may be a critical aspect of the response to DNA damage and prevention of cancer in higher eukaryotes. The fission yeast mutants will provide valuable tools for further investigation of the molecular mechanisms involved in this process.

Failure to recover from S phase arrest is associated with increased recombination

S phase arrest enhances loss rates of a non-essential minichromosome and elevates recombination in *rqh1* mutants. These results suggest that the role of *rqh1*$^+$ in recovery from S phase arrest could be to prevent excess recombination. High levels of recombination may explain the 'cut' phenotype of *rqh1*$^-$ cells after treatment with HU; if cells enter mitosis with sister chromatids entangled by unresolved recombination intermediates, subsequent chromosome segregation would be difficult or impossible. This model is consistent with studies showing that certain types of entangled chromosomes are not sensed by cell cycle checkpoints. For example, *S.pombe* and *S.cerevisiae* mutants lacking topoisomerase II also 'cut', as mitosis is initiated even though sister chromatids are catenated (Uemura and Yanagida, 1984; Holm *et al*., 1985; Uemura *et al*., 1987).

rqh1$^+$ could prevent recombination by a number of mechanisms. It could be a negative regulator of recombinases, with a particularly important function during S phase arrest, when a considerable amount of recombinogenic single-stranded DNA is present in the cell. Alternatively, the absence of *rqh1*$^+$ could cause DNA damage, leading to the generation of recombinogenic lesions. For example, it has been proposed that the RecQ helicases function together with topoisomerases to resolve replication intermediates at the end of S phase (Rothstein and Gangloff, 1995). It will be important to investigate interactions between *rqh1*$^+$ and *S.pombe* topoisomerases, particularly since the 'cut' phenotype observed after HU treatment resembles the phenotype caused by reduced topoisomerase II function (Uemura and Yanagida, 1984, 1986; Uemura *et al*., 1987).

The role of DNA helicase enzymatic activity in the regulation of recombination by the RecQ-related helicases remains to be determined. Although Sgs1 has been shown to be a helicase biochemically, an *S.cerevisiae sgs1* mutant lacking helicase activity is wild-type with regard to its interactions with *top1* and *top3* mutants (Lu *et al*., 1996). However, the effect of these mutations on recombination is not known. Possibly helicase activity may be required for some but not all of the *in vivo* functions of these proteins.

Control of recombination by a conserved family of RecQ helicase-related proteins

Based on structural features, *rqh1*$^+$ encodes the fourth member of a subfamily of putative DNA helicases related to the product of the *E.coli RECQ* gene. In addition to the helicase domain, the four members of this subfamily are approximately the same size and have other structural similarities. The other members of the subfamily are the human Blm and Wrn proteins and the *S.cerevisiae* Sgs1 protein (Figure 1). *BLM*$^-$, *sgs1*$^-$ and *rqh1*$^-$ cells show enhanced levels of genetic exchange, suggesting that the function of the RecQ-related proteins has been conserved during evolution. Loss of *BLM* function is associated with increased rates of carcinogenesis, suggesting that proper regulation of genetic exchange plays a vital role in the maintenance of genetic integrity. The Wrn protein also plays a role in maintenance of the genome and tumor prevention, although it may not regulate genetic exchange, as SCE is not elevated in *WRN*$^-$ cells (Bartram *et al*., 1976; Darlington *et al*., 1981; Gebhart *et al*., 1988).

Although both *SGS1* and *rqh1*$^+$ prevent recombination, loss of the gene has different biological consequences in the two yeasts, as *rqh1*$^-$ fission yeast are UV sensitive (Figure 2) and *sgs1*$^-$ cells are not (Watt *et al*., 1996). As we show here, the fission yeast *rqh1*$^+$ gene also plays a vital role in recovery from S phase arrest, while *SGS1* is not known to function in cell cycle control. Thus each yeast provides a unique opportunity to investigate the consequences of unregulated genetic exchange and, there-

fore, to understand the function of this new class of tumor suppressors.

Materials and methods

Genetic and molecular methods

Plasmids and strains were constructed using standard techniques (Sambrook *et al*., 1989; Moreno *et al*., 1991). All strains and plasmids used in this report are listed in Table I. Unless otherwise stated, 'wild-type' refers to strain TE271, which is 972 h^-; '*rqh1-h2*' refers to strain TE232 and '*rqh1*Δ' refers to strain TE767 (see Table I). *Schizosaccharomyces pombe* medium was prepared as previously described (Moreno *et al*., 1991).

Molecular analysis of *rqh1*$^+$

The *rqh1-h2* (*hus2-22*) strain was transformed with the *S.pombe* genomic libraries pURSP1 and pURSP2 (Barbet *et al*., 1992) and clones that complemented the radiation sensitivity of this strain were isolated, as described in Murray *et al.* (1992). Two independent overlapping clones were isolated, one of ~4850 bp (rqh1A) and one of ~6000 bp (rqh1B). 5151 bp of the rqh1B insert was sequenced using nested deletions and shown to contain an open reading frame of 3987 bp with no introns. Subsequently this sequence was reported by the Sanger Center *S.pombe* genome sequencing project, accession No. Q09811. Our sequence, which agrees with that from the genome project, has also been submitted to the DDBJ/EMBL/GenBank database, accession No. Y09426.

Sequence alignments of the predicted *rqh1*$^+$ gene product with other members of the RecQ-like DNA helicase family were carried out using the DNASTAR Megalign program. Similarities between the helicase domains of the members of this family were calculated using the GCG Bestfit program.

Construction of the rqh1 deletion mutant

The disruption construct was created by replacing the 3.6 kb *Nhe*I–*Age*I fragment of rqh1B (see Figure 2A) with a linker containing a *Not*I site:

```
5′-CTAGCGGCCGCA
      GCCGGCGTGGCC-3′
```

This construct was unable to complement the HU sensitivity of the *rqh1-h2* allele. An insert bearing the *rqh1* deletion was removed from the pUR19 vector by digestion with *Sac*I and *Sph*I and ligated into *Sac*I and *Sph*I digested pUC19. The 1.7 kb *ura4*$^+$ gene was then inserted into the *Not*I digested *rqh1* deletion construct as described previously (Barbet *et al*., 1992). This plasmid (pTE436, see Table I) was digested with *Sac*I and *Sph*I and the linear disruption construct, consisting of the *ura4*$^+$ gene flanked by *rqh1*$^+$ sequences, was isolated. This DNA was used to disrupt one copy of the *rqh1*$^+$ gene by the one-step disruption method in an h^+/h^+ *ura4-D18*/*ura4-D18* stable diploid (TE480, see Table I; a generous gift from G.Cottarel). The homologous integration event was confirmed by Southern blotting. This strain was crossed to an h^-/h^- *ura4-D18*/*ura4-D18* stable diploid (TE558, see Table I) to generate a sporulating diploid heterozygous for the *rqh1* deletion. These diploids were sporulated, tetrads were dissected and haploids that were HU sensitive and *ura*$^+$ were identified. The HU-sensitive and *ura*$^+$ phenotypes were found to co-segregate and to segregate 2:2 in all of the tetrads analyzed. One such haploid was picked for further analysis and Southern blot analysis was used to confirm deletion of the *rqh1*$^+$ gene (TE767, see Table I). Further crosses established that *rqh1*Δ was allelic to *rqh1-h2*.

Analysis of HU and UV response

UV and HU sensitivity were determined as previously described (Al-Khodairy and Carr, 1992; Enoch *et al*., 1992). Cells were fixed for microscopy and analyzed for 'cut' formation as previously described (Enoch *et al*., 1992). Unless otherwise noted, cells were grown on rich medium at 29°C. The number of colonies growing on two plates was counted for each viability measurement and each number was expressed as a proportion of the number of viable cells for that cell type at the start of the experiment. In every case the starting measurement corresponded to at least 480 cells. To investigate cell number increase in the presence of HU, cultures were grown to early log phase and HU was added to a final concentration of 10 mM. Samples were removed at the indicated times for analysis of cell number using a hemocytometer and scoring of 'cuts'. To examine 'cut' formation after UV irradiation, 1×10^7 cells from an early log phase culture were plated on each of two 23 cm^2 plates. The plates were irradiated at 150 J/m^2 and cells were then washed off the plates using 20 ml pre-warmed medium and incubated at 29°C. Samples were removed and examined microscopically for 'cuts'. To analyze recovery from S phase arrest (Figure 4), cultures were grown to early log phase and then arrested by the addition of HU to a final concentration of 10 mM. Cells were incubated for 4 h, filtered onto 0.45 μM HA filters (Millipore, Bedford, MA) and resuspended in fresh medium. Samples were removed at the indicated time points for FACS analysis (Shazer and Sherwood, 1990) and scoring of 'cuts'.

Determination of chromosome loss rates

The rate of loss of Ch^{16} (Niwa *et al*., 1986) from wild-type and *rqh1*$^-$ cells under normal growth conditions was calculated using two different methods. In the first method, chromosome loss was measured in the progeny of a single *ade*$^+$ cell after a known number of generations. In the second method, the increase in the number of cells that had lost Ch^{16} in a population of cells was determined after approximately one generation.

For the first method, TE786 and TE788 strains (see Table I) were grown in the absence of adenine to select for the presence of Ch^{16} and then streaked onto YE plates. After 3 days growth at 29°C, whole colonies of cells were dispersed in 1 ml YE medium. Cell number was measured using a hemocytometer and used to calculate the total number of cells in the colony, which can be used to determine the number of generations since the original *ade*$^+$ progenitor. The cells were then diluted into YE medium, sonicated briefly and plated onto YE plates at a concentration of ~1000 cells/plate. We did not see any difference between the growth rates of *ade*$^+$ and *ade*$^-$ cells on YE plates. After 2.5–3 days growth at 29°C the cells were replica-plated onto EMM plates supplemented with 7 μg/ml adenine, on which *ade*$^-$ cells are a pink color. Two days later the proportion of *ade*$^-$ colonies was calculated. Only those colonies which were completely pink (and had therefore arisen from a single *ade*$^-$ progenitor cell) were scored. For each wild-type colony dispersed in YE medium, ~37 000 colonies were screened for their *ade* phenotype; for each *rqh1*Δ colony, ~6000 colonies were screened. Rates of loss of Ch^{16} were determined for two wild-type colonies and 10 *rqh1*Δ colonies.

For the second method, cultures of cells were grown under selective conditions to ensure maintenance of Ch^{16} to an OD_{595} of 0.05–0.15. The cells were then filtered and resuspended in an equal volume of YE medium and incubated for 6 h. Samples were removed at the beginning and the end of the incubation period and cell number and the proportion of *ade*$^-$ segregants was determined as described above. In each of two experiments ~37 000 wild-type colonies were counted from the first time point and 40 000 from the last time point, and 37 000 *rqh1*Δ colonies were counted from the first time point and 15 000 from the last time point.

The rate of loss of Ch^{16} in each type of experiment was calculated using the following formula, adapted from Murakami *et al.* (1995).

$$\text{rate of loss} = 1-e^{(1/n)\ \ln R_n/R_0}$$

where R_0 and R_n are the proportion of *ade*$^+$ cells 0 and *n* generations after removal of selection respectively. In experiments using a single colony, R_0 was taken as 1, since the single progenitor cell for each colony was *ade*$^+$.

The rates of loss of the minichromosome from wild-type cells using the first method were calculated as 1.2×10^{-4} and 5.0×10^{-5} for each of two separate experiments, those using the second method were 1.2×10^{-4} and 2.5×10^{-4} for each of two separate experiments. We believe that differences in the rates between individual experiments are due to experimental error and are not significant. This is because the rates of loss of the minichromosome from *rqh1*Δ cells in the absence of HU calculated using the first method described above vary between 6.9×10^{-4} and 3.36×10^{-3}, even though each experiment was carried out using identical strains and techniques. Thus the two different methods of calculating the rate of loss of the minichromosome from cells under normal growth conditions give essentially the same results.

The overall loss rate was taken as the median value from 12 individual experiments for *rqh1*Δ cells and four individual experiments for wild-type cells.

To determine chromosome loss rates after incubation in HU, cells were grown under selective conditions to early mid log phase as described above, filtered and resuspended in rich medium (time 0). HU was then added to the cultures to a final concentration of 10 mM and they were incubated at 29°C. After 4 h the cells were filtered again, resuspended in fresh medium without HU and incubated for a further

5 h (time *n*). Samples were removed at time 0 and time *n* and cell number and chromosome loss rates were measured as described above. Cell number was also determined in samples before and after each filtration step, so that final measurements of cell number increase could be corrected for losses that occurred during filtration. In each of two experiments ~37 000 wild-type colonies were counted from time 0 and 40 000 from time *n*; 37 000 *rqh1*Δ colonies were counted from time 0 and 13 000 from time *n*. The rate of chromosome loss from the HU-treated cells was calculated using the formula described above. The number of generations, *n*, was calculated from the cell number increase between time 0 and time *n*, taking account of any change in dilution that occurred at the filtration step. The overall loss rate for HU-treated wild-type and *rqh1*Δ cells was taken to be the average of the loss rates determined in two separate experiments.

Measurement of recombination rates

The rate of mitotic recombination was calculated for the interval between two different *ade6* mutations; *ade6-M26*, which is a G→T substitution at G135, and *ade6-L469*, which is a C→T substitution at C1467. Reciprocal crossing over between these heteroalleles within this interval will lead to one copy of the *ade6* gene with both mutations and one wild-type copy of *ade6*, and the cell with therefore become *ade*$^+$. In order to calculate the rate of recombination under normal growth conditions, strain TE730 was crossed with strain TE744 and strain TE728 was crossed with strain TE725 (see Table I). The presence of the *mat2-102* allele in TE728 and TE730 prevents sporulation of the resulting diploids. Conjugants (TE747 and TE745 respectively, see Table 1) were streaked onto EMM plates supplemented with adenine, but not histidine or leucine; under these conditions only diploids resulting from conjugation can grow. After 3 days growth at 29°C, whole colonies of cells were dispersed in 330 µl YE medium. The number of cells in the colony (and therefore the number of generations that the single diploid progenitor cell had gone through) was estimated by determining cell number using a hemocytometer. To measure *ade*$^+$ recombinants, samples were plated on EMM plates supplemented with 50 mg/l guanine, which prevents residual growth of *ade*$^-$ colonies (Grossenbacher-Grunder and Thuriaux, 1981). Aliquots were also diluted and plated onto EMM plates supplemented with adenine to determine the total number of viable cells. All the plates were allowed to grow at 29°C for 3–5 days and the number of colonies on each plate was counted. The rate of recombination per generation for these cells was calculated using the formula given in the previous section, except under these circumstances R_0 and R_n are the proportion of *ade*$^-$ cells 0 and *n* generations after the start of the experiment. Under the conditions described above R_0 is 1, since the single progenitor cell for each colony was *ade*$^-$. Rates were calculated for eight diploid wild-type colonies and 10 diploid *rqh1*$^-$ colonies and the median value for each cell type was taken as the overall recombination rate.

The rate of recombination in cells treated with HU was determined as follows. Colonies of cells from freshly constructed diploids were grown in EMM supplemented with adenine to an OD_{595} of 0.1–0.25 (time 0). HU was then added to the culture to a final concentration of 10 mM and the cells were incubated at 29°C for a further 4 h (time *n*). Aliquots were removed from the cultures at time 0 and time *n* and percent recombinants was determined as described above. The rate of recombination per generation for these cells was calculated using the formula given in the previous section, where R_0 and R_n are the proportion of *ade*$^-$ cells at time 0 and *n* respectively. The recombination rate in HU was calculated for four diploid wild-type and five diploid *rqh1*$^-$ cultures and the median value was taken as the recombination rate in HU for that cell type.

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