

A checkpoint control linking meiotic S phase and recombination initiation in fission yeast

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During meiosis, high levels of recombination initiated by DNA double-strand breaks (DSBs) occur only after DNA replication. However, how DSB formation is coupled to DNA replication is unknown. We examined several DNA replication proteins for a role in this coupling in *Schizosaccharomyces pombe*, and we show that ribonucleotide reductase, the rate-limiting enzyme of deoxyribonucleotide synthesis and the target of the DNA synthesis inhibitor hydroxyurea (HU) is indirectly required for DSB formation linked to DNA replication. However, in cells in which the function of the DNA-replication-checkpoint proteins Rad1p, Rad3p, Rad9p, Rad17p, Rad26p, Hus1p, or Cds1p was compromised, DSB formation occurred at similar frequencies in the absence or presence of HU. The DSBs in the HU-treated mutant cells occurred at normal sites and were associated with recombination. In addition, Cdc2p is apparently not involved in this process. We propose that the sequence of meiotic S phase and initiation of recombination is coordinated by DNA-replication-checkpoint proteins.

cell cycle | DNA replication | meiosis | double-strand break formation

During meiosis, DNA replication occurs, followed by two rounds of chromosome segregation (1, 2). A high level of homologous recombination occurs after meiotic DNA replication but before the first meiotic nuclear division (1, 3–7). Meiotic recombination is required for the proper segregation of homologs at meiosis I, because a chromatid of one homolog recombines with a chromatid of the other homolog, thereby joining homologous chromosomes.

It is well established that meiotic recombination in both budding and fission yeasts is initiated by DNA double-strand breaks (DSBs), catalyzed by the DNA topoisomerase type-2-related enzyme Spo11 in budding yeast (8) and Rec12p in fission yeast (6). Both Spo11 in budding yeast and Rec12p in fission yeast are also essential for meiotic recombination. In addition to Rec12p, meiotic DSB requires multiple gene products that are also essential for meiotic recombination in two yeasts (1, 2).

The DNA-replication checkpoint operates during both the mitotic cell cycle and meiosis to ensure that chromosome segregation is blocked if DNA replication is incomplete (9–12). The proteins Rad1p, Rad3p, Rad9p, Rad17p, Rad26p, Hus1p, and Cds1p are required for operation of the DNA-replication checkpoint in fission yeast (9–11, 13). The DNA-replication-checkpoint proteins send a signal to block mitosis via the cyclin-dependent kinase Cdc2p complexed with the B-type cyclin Cdc13p (9). The Cdc2p-Cdc13p protein kinase is fully activated at the onset of mitosis by dephosphorylation of Cdc2p Tyr-15 (9). Phosphorylation of Cdc2p on Tyr-15 is catalyzed by both the Wee1p and Mik1p Tyr kinases, and dephosphorylation is carried out mainly by the Cdc25p Tyr phosphatase (14).

It has been proposed that DNA replication is directly coupled to initiation of DSB formation in budding yeast, because inhibition of the completion of DNA replication either by hydroxyurea (HU) treatment or by mutation of both the *clb5* and *clb6* genes (which encode B-type cyclin partners of Cdc28, a homolog of the cyclin-dependent kinase Cdc2p of fission yeast) blocks the formation of DSBs (4, 5). In addition, it has been reported that

the removal of active replication origin delays both replication and DSB appearance in budding yeast (5). However, in fission yeast, DSB formation occurs when completion of DNA replication is prevented by the inactivation of initiators of DNA replication (15).

To investigate whether this discrepancy between budding and fission yeasts might result from different ways of preventing meiotic DNA replication, we searched for DNA replication mutants of fission yeast that manifest a reduced level of meiotic DSB formation. Here, we show that ribonucleotide reductase (RNR), the rate-limiting enzyme of deoxyribonucleotide synthesis and the target of the DNA synthesis inhibitor HU, is indirectly required for DSB formation linked to DNA replication. In addition, we present evidence that the sequence of meiotic S phase and initiation of recombination is coordinated by DNA-replication-checkpoint proteins.

Materials and Methods

Fission Yeast Strains and Methods. All strains were constructed by standard procedures (16). The original *rad50S* strain was kindly provided by E. Hartsuiker (University of Sussex, Brighton, United Kingdom). The strains used for this study are given in Table 1.

Pulsed-Field Gel Electrophoresis (PFGE) and Southern Blot Analysis. Samples were prepared for PFGE as described (15). PFGE was performed in 0.8% agarose (chromosomal grade) with a CHEF DR II apparatus (Bio-Rad) for 43–48 h at 2 V/cm and 8–12°C in TAE buffer (Tris·acetate/EDTA) buffer with a switch time of 30 min. For Southern blot analysis, agarose plugs were prepared, and the DNA was digested with *NotI* and fractionated by PFGE before hybridization (17). The ³²P-labeled probes (≈1 kb) were prepared by the PCR with *Schizosaccharomyces pombe* genomic DNA as the template and 20-nt primers. The probe for the telomere-proximal part of the *NotI* J fragment of chromosome 1 spanned nucleotides 24,636–25,586 on cosmid c227 (GenBank accession no. AL133156). The probe for *rae1*⁺ on chromosome 2 spanned nucleotides 30,966–32,220 on cosmid c16A3 (GenBank accession no. AL021748). Sizes of DNA fragments were estimated from *Saccharomyces cerevisiae* chromosomal DNA (CHEF DNA Size Standard, Bio-Rad) and Low-Range PFG Markers (New England Biolabs) stained with ethidium bromide.

Quantitation of Meiotic DSB Frequency. The analysis of Southern blots of *rad50S* cell DNA was performed with a BIOIMAGE ANALYZER BAS1800 (Fuji Photo Film). A background level of radioactivity from the lightest part of each lane was subtracted from each value, and the total remaining level of radioactivity for each lane was normalized relative to the highest value.

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Abbreviations: DSB, double-strand break; RNR, ribonucleotide reductase; HU, hydroxyurea; PFGE, pulsed-field gel electrophoresis; mbs, meiotic break site.

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Table 1. Strains used in this study

Strain	Genotype
HM34	<i>h⁻ pat1-114 leu1-32 (pat1)</i>
HM113	<i>h⁺ pat1-114 cdc2-L7 (pat1 cdc2-L7)</i>
HM336	<i>h⁻/h⁻ pat1-114/pat1-114 rec12::LEU2/rec12::LEU2 leu1-32/leu1-32 ade6-M210/ade6-M216 (pat1 rec12)</i>
HM338	<i>h⁺ pat1-114 orp1-4 (pat1 orp1)</i>
HM605	<i>h⁻/h⁻ pat1-114/pat1-114 mik1::ura4/mik1::ura4 wee1-50/wee1-50 ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216 (pat1 mik1 wee1)</i>
HM713	<i>h⁻ pat1-114 rad9-192 (pat1 rad9)</i>
HM731	<i>h⁻ pat1-114 rad17-h21 leu1-32 (pat1 rad17)</i>
HM783	<i>h⁻ pat1-114 rad26::ura4 ura4-D18 leu1-32 (pat1 rad26)</i>
HM798	<i>h⁻ pat1-114 hus1-14 leu1-32 (pat1 hus1)</i>
HM1307	<i>h⁺/h⁺ pat1-114/pat1-114 ade6-M210/ade6-M216 (pat1)</i>
HM1482	<i>h⁺ pat1-114 rad1::ura4 ura4-D18 leu1-32 (pat1 rad1)</i>
HM1691	<i>h⁻ pat1-114 rad50S-kan^r leu1-32 (pat1 rad50S)</i>
HM1801	<i>h⁻ pat1-114 cds1::ura4 rad50S-kan^r ura4-D18 leu1-32 (pat1 cds1 rad50S)</i>
HM1804	<i>h⁺ pat1-114 rad50S-kan^r mik1::ura4 wee1-50 ura4-D18 leu1-32 (pat1 mik1 wee1 rad50S)</i>
HM1805	<i>h⁺ pat1-114 rad50S-kan^r rec12::LEU2 leu1-32 (pat1 rec12 rad50S)</i>
HM1824	<i>h⁻ pat1-114 rad50S-kan^r mik1::ura4 wee1-50 rec12::LEU2 ura4-D18 leu1-32 (pat1 mik1 wee1 rec12 rad50S)</i>
HM1842	<i>h⁻ pat1-114 cds1::ura4 rad50S-kan^r rec12::LEU2 ura4-D18 leu1-32 (pat1 cds1 rec12 rad50S)</i>
HM1936	<i>h⁻/h⁻ pat1-114/pat1-114 cdc22-M45/cdc22-M45 ura4-D18/+ leu1-32/leu1-32 ade6-M210/ade6-M216 (pat1 cdc22)</i>
HM2690	<i>h⁻ pat1-114 rad3-ts rad50S-kan^r leu1-32 (pat1 rad3 rad50S)</i>
HM2699	<i>h⁻ pat1-114 rad3-ts rec12::LEU2 rad50S-kan^r leu1-32 (pat1 rad3 rec12 rad50S)</i>
HM2849	<i>h⁺/h⁺ pat1-114/pat1-114 rad3-ts-kan^r/rad3-ts-kan^r rec12::LEU2/rec12::LEU2 leu1-32/leu1-32 ade6-M210/ade6-M216 (pat1 rad3 rec12)</i>
HM2877	<i>h⁺/h⁺ pat1-114/pat1-114 rad3-ts/rad3-ts leu1-32/leu1-32 ade6-M210/ade6-M216 (pat1 rad3)</i>
HM3696	<i>h⁺/h⁺ pat1-114/pat1-114 rad50S-kan^r/rad50S-kan^r leu1-32/+ ade6-M210/ade6-M216 (pat1 rad50S)</i>
HM4065	<i>h⁺ pat1-114 rad3-136 cdc2-22 leu1-32 (pat1 rad3 cdc2-22)</i>
HM4066	<i>h⁺ pat1-114 cdc2-22 leu1-32 (pat1 cdc2-22)</i>
HM4082	<i>h⁺ pat1-114 rad3-136 rec12::LEU2 cdc2-22 leu1-32 (pat1 rad3 rec12 cdc2-22)</i>
HM4088	<i>h⁺ pat1-114 rad3-ts cdc13::ura4⁺-pREP41-cdc13⁺ rad50S-kan^r ura4-D18 leu1-32 (pat1 rad3 cdc13 rad50S)</i>
HM4090	<i>h⁺ pat1-114 cdc13::ura4⁺-pREP41-cdc13⁺ rad50S-kan^r ura4-D18 leu1-32 (pat1 cdc13 rad50S)</i>
HM4130	<i>h⁺ pat1-114 rad3-ts cdc13::ura4⁺-pREP41-cdc13⁺ rec12::LEU2 rad50S-kan^r ura4-D18 leu1-32 (pat1 rad3 rec12 cdc13 rad50S)</i>
HM4132	<i>h⁺ pat1-114 cdc13::ura4⁺-pREP41-cdc13⁺ rec12::LEU2 rad50S-kan^r ura4-D18 leu1-32 (pat1 rec12 cdc13 rad50S)</i>
HM4133	<i>h⁺ pat1-114 cdc2-22 rec12::LEU2 leu1-32 (pat1 rec12 cdc2-22)</i>

Other Techniques. Meiosis was induced as described in ref. 12. Samples for flow-cytometric analysis were prepared and DNA content was determined with a FACScan (Becton Dickinson) also as described (12).

Results and Discussion

To investigate which DNA replication protein is required for DSB formation in fission yeast, we constructed double temperature-sensitive (ts) mutants of various DNA replication protein genes and *pat1*, which encodes a negative regulator of meiosis whose inactivation by a ts mutation synchronously induces meiosis regardless of its ploidy and high levels of recombination (18). In addition, the frequency of meiotic DSB formation in haploid strains is similar to that in diploid strains in *pat1*-induced meiosis (ref. 17 and this study). Cells were synchronized in G₁ phase of the cell cycle by nitrogen deprivation and shifted to the restrictive temperature of 36°C to induce meiosis. Meiotic DNA replication was largely complete 3 h after the temperature shift

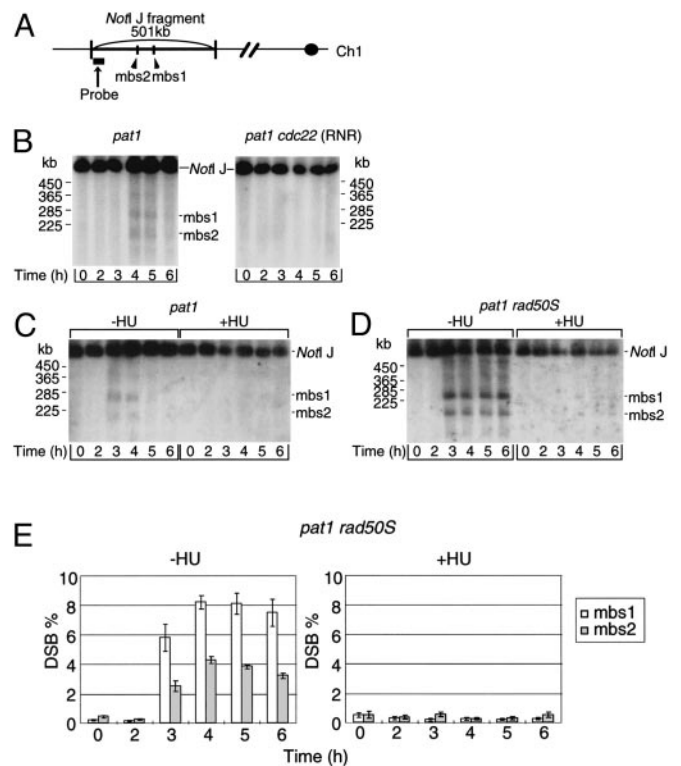


Fig. 1. RNR is required for DSB formation. (A) Position of the probe corresponding to the telomere-proximal portion of the *NotI* J fragment of chromosome 1. (B) *pat1* (HM1307) and *pat1 cdc22* (HM1936) cells were grown and then transferred to nitrogen-free medium for 16 h at 24°C to arrest cells in G₁ phase. Meiosis was induced by shifting the temperature to 36°C at time 0. Samples were subjected to digestion of DNA with *NotI* and then analyzed by PFGE and Southern blot hybridization with the probe described above. (C and D) *pat1* (HM1307) (C) and *pat1 rad50S* (HM1691) (D) cells were arrested in G₁ phase, and meiosis was induced at 34°C in the absence (–) or presence (+) of 24 mM HU. Samples collected at the indicated times were analyzed as in B. (E) Quantitation of meiotic DSB frequency at *mbs1* and *mbs2* determined from experiments similar to that shown in D. Data are means ± SE of values from three independent experiments.

in *pat1* cells, as judged by flow-cytometric analysis (Fig. 6, which is published as supporting information on the PNAS web site). Meiotic DSBs were detected in these cells by Southern blot analysis with a telomere-proximal part of the *NotI* J fragment of chromosome 1 as a probe (Fig. 1 A and B) (17). Bands corresponding to the prominent meiotic break site (*mbs1*) and *mbs2* were detected in *pat1* cells in addition to several minor bands (Fig. 1B) (17). One of the DNA replication mutants, *cdc22*, manifested a defect in DSB formation (Fig. 1B), and most meiotic DNA replication was blocked in these cells (Fig. 6). Cdc22p is the large subunit of RNR in fission yeast (19). A relatively high temperature (36°C) was required to inactivate *cdc22* in the ts mutant. Given that DNA replication is delayed and the efficiency of sporulation is reduced at this temperature (15), we used HU to inhibit RNR and a temperature of 34°C to induce meiosis efficiently. Treatment with HU inhibited DNA replication (Fig. 6) and reduced the intensity of bands corresponding to *mbs1* and *mbs2* in *pat1* cells to a level similar to that observed in *pat1 rec12* cells (Figs. 1C and 6) (17). Analysis with the *rae1*⁺ fragment of chromosome 2 as a probe also revealed that DSB formation was blocked efficiently by HU (Fig. 7, which is published as supporting information on the PNAS web site).

We next examined a *rad50S* mutant in which the repair of DSBs is prevented and with which it is possible to obtain a quantitative estimate of a low level of DSBs (17). HU treatment

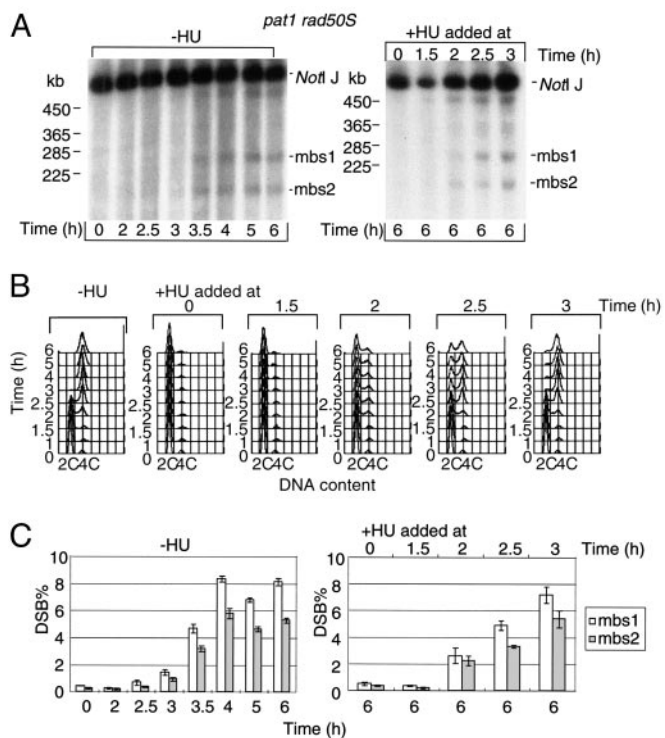


Fig. 2. HU treatment inhibits DSB formation by acting in meiotic S phase. (A) *pat1 rad50S* (HM3696) cells were arrested in G₁ phase, and meiosis was induced in the absence (–) or presence (+) of HU, with the exception that HU was added at the indicated times after the shift to 34°C. Samples were subjected to PFGE, as described for Fig. 1B. The probe used was the one as shown in Fig. 1A. (B) DNA content of the cells in A was determined by flow cytometry. (C) Quantitation of meiotic DSB frequency at mbs1 and mbs2 determined from experiments similar to that shown in A. Data are means ± SE of values from four independent experiments.

effectively blocked DNA replication and reduced DNA fragments including those corresponding to breakage at mbs1 and mbs2 to virtually undetectable levels in *pat1 rad50S* cells (Figs. 1D and 6). The frequency of DSB formation at mbs1 or mbs2 at 6 h in *pat1 rad50S* cells was ≈7% and 4%, respectively, in the absence of HU and <0.5% at both sites in the presence of HU, the latter frequency being similar to that observed in *pat1 rec12 rad50S* cells (data not shown) or the background level (Figs. 1E and 6) (17). These results were confirmed with the *rae1*⁺ probe (Fig. 7). Together, our findings suggested that RNR is directly or indirectly required for DSB formation, consistent with observations in budding yeast (5).

If HU directly inhibited meiotic DSB formation, the latter would be expected to be blocked by HU treatment even after meiotic DNA replication. We added HU to the culture medium of *pat1 rad50S* cells before, during, or after meiotic DNA replication. The formation of DSBs was blocked almost completely only when HU was added before meiotic DNA replication at 0 or 1.5 h after the induction of meiosis (Fig. 2A and B). After quantitative analysis of DSB formation (Fig. 2C), we found that the proportion of DSB formation was correlated with DNA replication. Thus, these results indicate that HU does not inhibit DSB formation directly but that HU achieves this effect through inhibition of meiotic DNA replication.

Because the DNA-replication checkpoint couples DNA replication and nuclear division, it is possible that this checkpoint also operates to coordinate meiotic DNA replication and DSB formation. We investigated whether Rad3p is required for prevention of DSB formation in cells in which meiotic DNA

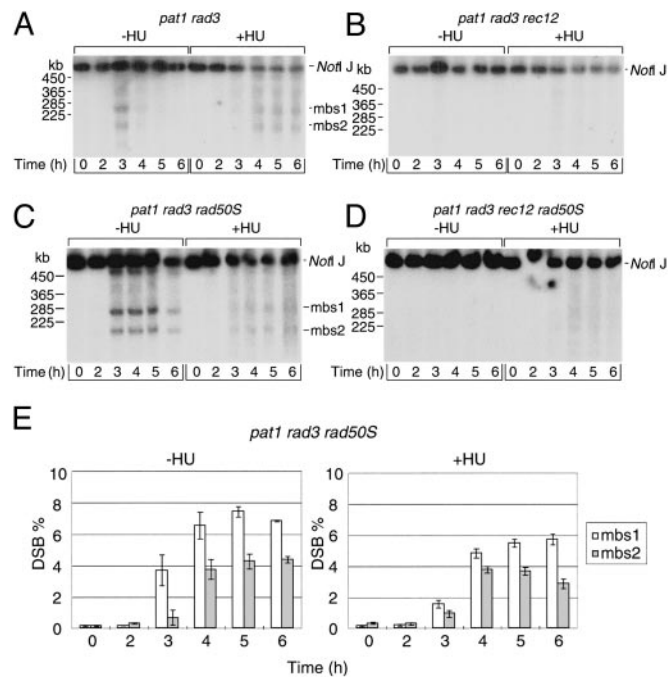


Fig. 3. Rad3p is required for coupling of meiotic DNA replication to DSB formation. (A–D) *pat1 rad3* (HM2877) (A), *pat1 rad3 rec12* (HM2849) (B), *pat1 rad3 rad50S* (HM2690) (C), or *pat1 rad3 rec12 rad50S* (HM2699) (D) cells were arrested in G₁ phase, and meiosis was induced at 34°C in the absence (–) or presence of HU (+), with HU added at time 0. Samples collected at the indicated times thereafter were analyzed as described for Fig. 1B. The same probe was used as in Fig. 1A. (E) Quantitation of meiotic DSB frequency at mbs1 and mbs2 for *pat1 rad3 rad50S* (HM2690) cells determined from experiments similar to that shown in C. Data are means ± SE of values from three independent experiments.

replication is incomplete by examining *pat1 rad3* double-mutant cells induced to undergo meiosis in the absence or presence of HU (Fig. 3). Mutant cells in which meiotic DNA replication was prevented by HU treatment (Fig. 8, which is published as supporting information on the PNAS web site) progressed through meiotic nuclear division (12) and manifested DSBs, although the appearance of the DSBs was delayed by 1 h and did not disappear (Figs. 3A and 8). To examine whether the DSBs observed in the presence of HU were generated by the normal meiotic DSB machinery, we constructed a *pat1 rad3 rec12* mutant. No specific DNA fragments were detected in this mutant after treatment with HU (Figs. 3B and 8), suggesting that most of the DSBs depend on Rec12p. These results also suggest that Rad3p negatively regulates DSB formation in a Rec12p-dependent manner. In addition, the same Rec12p-dependent prominent bands were detected with the *rae1*⁺ probe in *pat1 rad3* cells that had been incubated in the absence or presence of HU (Fig. 8). Thus, most of the DSBs in the *pat1 rad3* cells treated with HU appeared not to be attributable to replication fork stalling, given that the collapse of stalled replication forks would produce DSBs at sites other than those normally targeted and would be independent of Rec12p. We confirmed the above results by using the *rad50S* mutation (Figs. 3C and D and 8) and quantitated the frequency of DSB formation in these cells (Fig. 3E). The frequencies of DSB formation at mbs1 and mbs2 were ≈7% and 4%, respectively, in the absence of HU treatment, and ≈6% and 4% in HU-treated cells. Together, these findings suggest that Rad3p plays a major role in preventing DSB formation in HU-treated cells that are not mediated through replication fork stabilization whose function is found in the DNA-replication-checkpoint proteins (20).

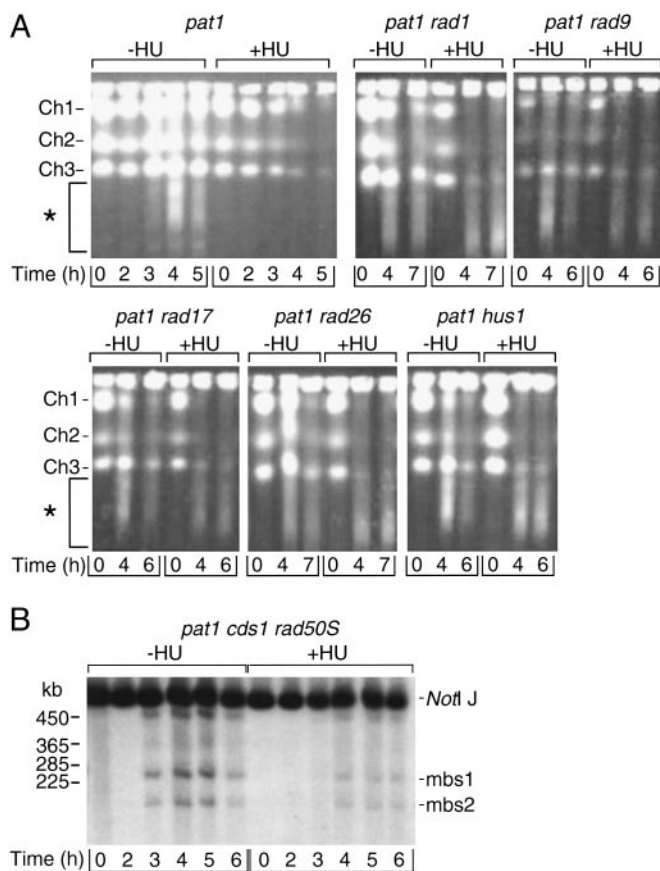


Fig. 4. The DNA-replication-checkpoint proteins are required for coupling of meiotic DNA replication to DSB formation. (A) *pat1* (HM34), *pat1 rad1* (HM1482), *pat1 rad9* (HM713), *pat1 rad17* (HM731), *pat1 rad26* (HM783), and *pat1 hus1* (HM798) cells were arrested in G₁ phase, and meiosis was induced at 34°C in the absence (-) or presence (+) of HU, with HU added at time 0. Samples collected at the indicated times thereafter were analyzed by PFGE, followed by ethidium bromide staining. Ch1, Ch2, and Ch3 indicated the positions of chromosomes 1 (5.7 Mb), 2 (4.6 Mb), and 3 (3.5 Mb), respectively. The smear (*) shows DSBs generated during meiosis. (B) *pat1 cds1 rad50S* (HM1801) cells were arrested in G₁ phase, and meiosis was induced at 34°C. Samples were analyzed as described for Fig. 1B. The probe used in B was the one shown in Fig. 1A.

To determine whether other DNA-replication-checkpoint proteins, including Rad1p, Rad9p, Rad17p, Rad26p, and Hus1p, are required for the coupling of DNA replication to DSB formation, we monitored the formation of DSBs in the corresponding DNA-replication-checkpoint mutants with or without HU. DSB formation in the presence of HU was observed in these mutants (Fig. 4A), indicating that all of these proteins are required for prevention of DSB formation when DNA replication is reduced by HU. We next monitored DSB formation and DNA replication in *pat1 cds1 rad50S* cells without or with HU (Fig. 4B and Fig. 9, which is published as supporting information on the PNAS web site). Formation of DSBs at both *mbs1* and *mbs2* was observed in *pat1 cds1 rad50S* cells in the presence of HU in a Rec12-dependent manner, indicating that Cds1p is also required for coupling of DNA replication to DSB formation (Figs. 4B and 9). This result was confirmed by using the *rae1*⁺ probe (Fig. 9).

In HU-treated cells in which DNA replication is incomplete, the DNA-replication-checkpoint proteins inhibit the kinase activity of Cdc2p by mediating its phosphorylation on Tyr-15, and thereby, they prevent entry into mitosis or the first meiotic nuclear division (9–11). If the block of DSB formation by HU

were mediated through Cdc2p, then inactivation of Cdc2p would be expected to prevent DSB formation even in *rad3* cells. We used a *cdc2-22* allele whose inactivation gives rise to cell-cycle arrest in G₂ only during the mitotic cell cycle (21). As expected, meiotic DNA replication occurred normally in *pat1cdc2-22* and *pat1 rad3 cdc2-22* cells (Fig. 10, which is published as supporting information on the PNAS web site). However, meiotic nuclear division was severely delayed in both of these cells. At 8 h, >60% of *pat1* or *pat1 rad3* cells entered meiosis II whereas <9% of *pat1 cdc2-22* or *pat1 rad3 cdc2-22* cells did. Rec12-dependent DSB formation occurred in *pat1 rad3 cdc2-22* cells without or with HU, whereas it was severely blocked in *pat1 cdc2-22* cells (Figs. 5 and 10). These results suggest that Cdc2p is not involved in coupling DNA replication and DSB formation. To confirm this hypothesis, we next examined a *cdc13*⁺ switch-off strain in which *cdc13*⁺ expression is inhibited by thiamine (22). Meiotic DNA replication occurred normally in *pat1 cdc13 rad50S* and *pat1 rad3 cdc13 rad50S* cells (Fig. 10), and at 8 h, >94% of these cells failed to enter the first meiotic nuclear division, whereas <8% of *pat1 rad50S* or *pat1 rad3 rad50S* cells did. DSB formation occurred in *pat1 rad3 cdc13 rad50S* cells depending on Rec12p without or with HU, whereas it was severely blocked in *pat1 cdc13 rad50S* cells (Figs. 5 C and D and 10). These results suggest that the major B-type cyclin Cdc13p is not required for DSB formation. If the block of DSB formation by HU was mediated through phosphorylation of Cdc2p on Tyr-15, then prevention of this phosphorylation would be expected to allow DSB formation even in the presence of HU. DSB formation occurred (although at a low level) in *pat1 mik1 wee1* and *pat1 mik1 wee1 rad50S* cells in the absence of HU (Fig. 11, which is published as supporting information on the PNAS web site). Treatment with HU prevented DNA replication and DSB formation in both of these cells (Fig. 11), even though Tyr-15 of Cdc2p is dephosphorylated and the kinase activity of Cdc2p is high in *pat1 mik1 wee1* cells (12). All of these findings suggest that the target of the DNA-replication checkpoint in this situation is something other than Cdc2p.

Our data lead us to propose the following model for the coupling of meiotic DNA replication and recombination initiation. Treatment of cells with HU results in the inactivation of RNR, leading to the inhibition of the bulk of DNA replication in meiotic S phase. The resulting change in DNA structure likely results in the activation of DNA-replication-checkpoint proteins, including Rad1p, Rad3p, Rad9p, Rad17p, Rad26p, and Hus1p, and this signal is then transmitted to Cds1p, which prevents the initiation of recombination through regulation of unidentified targets (possibly including Rec12p).

Mec1 (a homolog of fission yeast Rad3p) is not required for coupling of meiotic DNA replication to recombination initiation in budding yeast (5), although Mec1 is required for the linkage between meiotic DNA replication and the first meiotic nuclear division (23). This discrepancy might be due to the different roles of the checkpoint proteins in the two yeasts. Budding yeast Mec1 is essential for viability, whereas fission yeast Rad3p is not (11, 24). The hypomorphic mutant *mec1-1* is deficient only in coupling of DNA replication and meiotic nuclear division, the link between DNA replication and DSB formation being intact (23). It is also possible that these two yeasts rely on completely different systems for the coordination between meiotic S phase and recombination initiation. DSBs were detected in fission yeast cells deficient in the B-type cyclins Cig2p (data not shown) or Cdc13p (this study) but not in budding yeast *clb5* and *clb6* mutants (4). However, B-type cyclins are required for meiotic S phase in both yeasts (23, 25, 26). These observations suggest that the roles of B-type cyclins and this cyclin-dependent kinase during meiosis differ between the two yeast species (4, 15, 18, 23, 26).

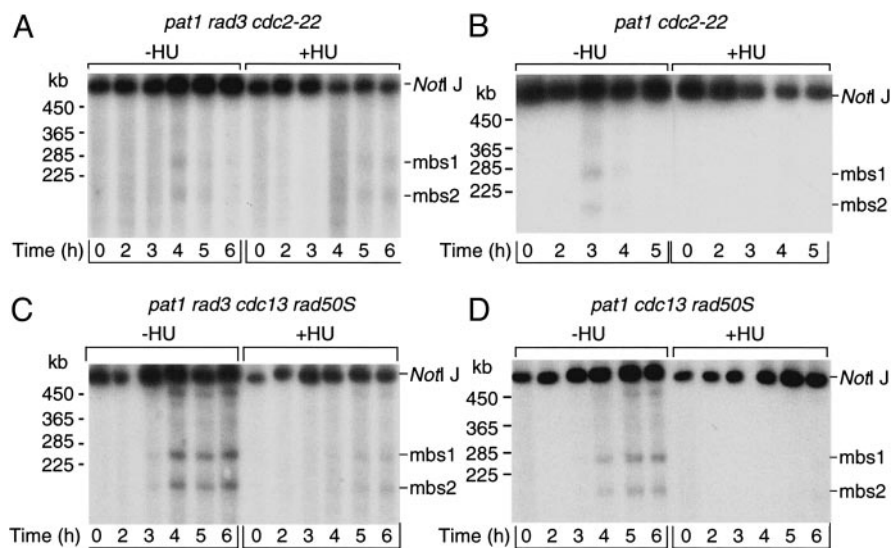


Fig. 5. Cdc2p/Cdc13p is not involved in coupling meiotic DNA replication to DSB formation. (A and B) *pat1 rad3 cdc2-22* (HM4065) (A) and *pat1 cdc2-22* (HM4066) (B) cells were arrested in G₁ phase, and meiosis was induced at 36.5°C in the absence (–) or presence (+) of HU, with HU added at time 0. Samples collected at the indicated times thereafter were analyzed by PFGE as in described for Fig. 1B. The probe used was the one shown in Fig. 1A. (C and D) *pat1 rad3 cdc13 rad50S* (HM4088) (C) and *pat1 cdc13 rad50S* (HM4090) (D) cells were arrested in G₁ phase, and meiosis was induced at 34°C in the absence or presence of HU, with HU added at time 0. Thiamine was added at time 0 to switch off *cdc13*⁺ expression. Samples collected at the indicated times thereafter were analyzed by PFGE and Southern blotting, as described above.

Compared with the results in ref. 15, Cdc22p inactivation is different from inactivation of other DNA replication initiation proteins that include Orp1p, Cdc18p, Cdc19p, and Cdc21p. Cdc22p inactivation or HU treatment inhibits DSB formation, whereas inactivation of these DNA replication proteins does not. We investigated the relation between Cdc22p and other DNA replication proteins. HU treatment significantly inhibited the DSB formation observed in *pat1 orp1* and *pat1 cdc2-L7* cells, whereas DNA replication was effectively blocked in both of these cells without or with HU as judged by flow-cytometric analysis (Fig. 12, which is published as supporting information on the PNAS web site) (15). There are at least three possibilities that cause these results. First, the degree of DNA replication may be important; that is, little replication is evident in the *cdc22* or HU treated cells, whereas a small amount of DNA replication has occurred in *orp1* and other DNA replication mutants (15). It is possible that a subtle difference of the degree of DNA replication may be important. Second, the absence of dNTPs in the HU-treated cells may have a physiological effect that is independent of the replication fork stalling. In mitotic cells, Chk1p is not required for an arrest in HU, but it is required for arrest

in the replication mutants (27). Third, the DNA structure that activates the checkpoint signal may play a role. The DNA structure may be different depending on the way of blocking DNA replication. The checkpoint signal that activates Cds1p is generated in the *cdc22* or HU treated cells but not in *orp1 cdc18* cells during the mitotic cell cycle (28). Therefore, if meiotic S phase is not initiated, DSB formation can, nonetheless, take place. The parallel conclusion for this study would then be that if meiotic S phase is initiated, but blocked, then DSB formation is delayed until replication is complete. Perhaps then, the corollary to this conclusion is that DSB formation occurs in the DNA replication initiation mutants because there is nothing (i.e., no unreplicated DNA) to prevent it. Furthermore, DSB formation is blocked only if an aberrant DNA structure arises that activates the checkpoint.

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