Rad3-Cds1 Mediates Coupling of Initiation of Meiotic Recombination with DNA Replication

Mei4-DEPENDENT TRANSCRIPTION AS A POTENTIAL TARGET OF MEIOTIC CHECKPOINT

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Premeiotic S-phase and meiotic recombination are known to be strictly coupled in Saccharomyces cerevisiae. However, the checkpoint pathway regulating this coupling has been largely unknown. In fission yeast, Rad3 is known to play an essential role in coordination of DNA replication and cell division during both mitotic growth and meiosis. Here we have examined whether the Rad3 pathway also regulates the coupling of DNA synthesis and recombination. Inhibition of premeiotic S-phase with hydroxyurea completely abrogates the progression of meiosis, including the formation of DNA double-strand breaks (DSBs). DSB formation is restored in rad3 mutant even in the presence of hydroxyurea, although repair of DSBs does not take place or is significantly delayed, indicating that the subsequent recombination steps may be still inhibited. Examination of the roles of downstream checkpoint kinases reveals that Cds1, but not Chk1 or Mek1, is required for suppression of DSB in the presence of hydroxyurea. Transcriptional induction of some rec+ genes essential for DSB occurs at a normal timing and to a normal level in the absence of DNA synthesis in both the wild-type and cds1Δ cells. On the other hand, the transcriptional induction of the mei4+ transcription factor and cdc25+ phosphatase, which is significantly suppressed by hydroxyurea in the wild-type cells, occurs almost to a normal level in cds1Δ cells even in the presence of hydroxyurea. These results show that the Rad3-Cds1 checkpoint pathway coordinates initiation of meiotic recombination and meiotic cell divisions with premeiotic DNA synthesis. Because mei4+ is known to be required for DSB formation and cdc25+ is required for activation of meiotic cell divisions, we propose an intriguing possibility that the Rad3-Cds1 meiotic checkpoint pathway may target transcription of these factors.

Checkpoints play a central role in coordinated progression of the cell cycle (reviewed in Ref. 1). Among them, monitoring of DNA replication is crucial for stable maintenance of the genome. Unscheduled arrest of DNA replication forks or incomplete replication of the genome elicits checkpoint signals that arrest the ongoing S-phase or inhibit mitosis. These signals involve ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) (Rad3 or Tel1 in fission yeast)-related kinases and their downstream Chk1 or Chk2 (Chk1 or Cds1 in fission yeast) kinases. It is known that inhibition of premeiotic DNA replication (meiS) by hydroxyurea (HU) leads to inhibition of meiosis I and II in a manner dependent on Rad3 and Cds1 (1, 2). Inhibition of Cdc2 by tyrosine 15 phosphorylation in the presence of HU is relieved by rad3 or cdc25+ mutation. Thus, checkpoint signaling similar to the one operating during mitosis regulates the coordination of DNA replication and cell division during meiosis.

High frequency recombination is an event specific to meiosis, and in budding yeast it is strictly coupled to ongoing DNA synthesis (3–5). It was reported that, in fission yeast, defect in meiotic recombination delays meiosis I and II in a manner dependent on Mek1 (6, 7), a meiosis-specific Cds1-related kinase. It has not been known whether premeiotic DNA replication and meiotic recombination are regulated under checkpoint regulation. Initiation of meiotic recombination is marked by the introduction of DNA double-strand breaks (DSBs) on the genome (8). Very recently, while we were preparing this report, it was reported that meiotic DSB formation in fission yeast is coupled to premeiotic DNA replication in a manner dependent on DNA replication checkpoint proteins (9). We have shown here that the Rad3-Cds1 pathway, but not Chk1 or Mek1 pathway, inhibits the generation of meiotic DSB when premeiotic DNA replication is inhibited by HU in fission yeast. We have also presented data suggesting that Rad3-Cds1 may target the transcription of the mei4+ transcription factor, which is required for DSB formation, for suppression of initiation of meiotic recombination in response to inhibition of DNA replication. We will discuss a possible novel mechanism of the Rad3-Cds1 meiotic checkpoint regulation.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media—Schizosaccharomyces pombe strains used in this study were JZ267 (h+ pat1–114 leu1 ade6-M210), KO192 (h+ pat1–114 chkl::ura4+ leu1–32 ade6 ura4-D18), KO194 (h+ pat1–114 cds1::ura4+ leu1–32 ura4-D18), KO273 (h+ pat1–114 rec12::ura4+ cds1::ura4+ leu1–32 ura4-D18), KO298 (h+ pat1–114 mekl::kanMX6 leu1–32 ade6-M210), and KO350 (h+ pat1–114 rad3::ura4+ leu1–32 ura4-D18). Cells were grown in minimal medium (MM) containing the required supplements (20 g/liter of glucose, 3 g/liter of potassium biphthalate, 2.2 g/liter of Na2HPO4, and 5 g/liter of NH4Cl). MM lacking NH4Cl was used for nitrogen starvation experiments. General genetic methods were previously described (10, 11).

Induction of Meiosis in pat1–114 Cells—Cells, grown at 25 °C to 5 × 106 cells/ml in MM supplemented with 100 μg/ml of leucine and 100 μg/ml of adenine, were washed with MM lacking NH4Cl four times and were resuspended in MM lacking NH4Cl supplemented with 25 μg/ml of leucine, followed by incubation for 16 h at 25 °C. After addition of an equal volume of prewarmed MM containing 1 g/liter of NH4Cl supplemented with 50 μg/ml of leucine, 70 μg/ml of adenine, and with or without 24 mM...
HU, meiosis was induced by shifting the temperature to 32 °C. Equal volumes of samples were withdrawn at each time point from each culture for FACS and DNA analyses on pulsed-field gel electrophoresis.

**Flow Cytometry**—5 x 10^6–1 x 10^7 cells were spun down, washed once with water, and then fixed in 70% ethanol. The fixed cells were treated with RNase A (0.1 mg/ml), stained with propidium iodide (2 μg/ml) as described previously (10), and processed for flow cytometry using FACScalibur (BD Biosciences).

**Northern Analyses of Transcription in Fission Yeast Cells**—Total RNA was prepared from synchronized pat1-114 or pat1-114 cds1Δ
cells as described previously (12). Ten μg of each total RNA was used for analysis. For probes, portions of open reading frames were amplified from genomic DNA by PCR for rec6, rec7, rec12, mei4, spo6, mdc2, mde3, and amp1 (loading control). For the cdc25 probe, the 0.9-kb BamHI-BglII fragment was used as described previously (13).

Detection of Meiotic DSBs by Pulsed-field Gel Electrophoresis—pat1–114 cells released from nitrogen starvation were harvested every hour and treated as described previously (14). Pulsed-field gel electrophoresis was carried out in a 0.6% chromosomal grade agarose gel (Bio-Rad) on Bio-Rad CHEF-DRIII apparatus, recirculated at 14 °C. Electrophoresis was for 48–72 h at 1.5 V/cm in 0.5× Tris-acetate-EDTA buffer with a switch time of 30 min at an included angle of 106°. For analyses by Southern hybridization, electrophoresis was conducted in a 0.8% chromosomal grade agarose gel (Bio-Rad) for 24 h at 6 V/cm in 0.5× Tris borate-EDTA buffer, with linearly ramped 60–120-s switch time at an included angle of 120° as described previously (8).

RESULTS

Suppression of DSB Formation in the Presence of HU Is Abrogated in rad3Δ Mutant—Meiotic recombination is initiated by the extensive induction of DSBs on chromosomal DNAs. DSBs can be detected by analyzing the chromosomal DNA on pulsed-field gel electrophoresis (8). To analyze the meiotic DSB, we have utilized pat1 temperature-sensitive cells in which meiotic processes can be induced synchronously by shifting up the temperature. pat1+ encodes a protein kinase that phosphorylates Mei2 protein and negatively regulates meiosis both at the stage of premeiotic DNA replication and at that of meiosis I. In pat1–114 cells, meiosis can be induced in a haploid state at a nonpermissive temperature in a synchronous manner (13, 15–17). During the pat1-induced meiosis, premeiotic DNA replication (meiS) initiates at 1–2 h after the shift and completes by 3 h (Fig. 1A). DSBs are detected at 3 h after the shift and disappear by 4–5 h (Fig. 1B). When HU is added at time 0, meiS is completely blocked and cells stay as one nuclear state. Under this condition, generation of DSBs is also completely suppressed. We have constructed rad3Δ pat1–114 strain and examined meiS and DSB. In the absence of HU, meiS and DSB occurred with identical or slightly accelerated timing. In the presence of HU, meiS is completely suppressed as in the rad3Δ cells but the DSBs are generated, albeit with significantly delayed timing (Fig. 1, A and B). The maximum level of DSBs is detected at 6 h after the shift. This indicates that suppression of meiotic DSB in the presence of HU requires Rad3 function. Persistent presence of DSBs in rad3Δ cells suggests that the subsequent repair of DSBs by recombination functions is still suppressed or extremely delayed even in the absence of the Rad3 function (Fig. 1B and supplemental Fig. S1).

Cds1 Checkpoint Kinase Is Required for Suppression of DSBs in the Presence of HU—Cds1 and Chk1 are known to function downstream of Rad3 in DNA replication and DNA damage checkpoint signaling in fission yeast (1). During meiosis, Mek1, a Cds1-related meiosis-specific kinase, was shown to be required for coupling of recombination and meiosis (6, 7). We have introduced each of these checkpoint kinase mutants into pat1–114 cells and examined meiS and DSB formation. In the absence of HU, all these mutants enter meiS with normal or slightly accelerated timing, completing it by 3 h. DSBs are also generated at a normal level, peaking at 3–4 h (Fig. 2A). In the presence of HU, meiS is suppressed in all the mutants. DSBs are suppressed in chk1Δ and mek1Δ cells as in the wild-type cells but are clearly induced in cds1Δ cells at a timing similar to that observed in rad3Δ cells (Figs. 1A and 2A; see also supplemental Fig. S1). To prove that the fragmented DNAs observed are indeed the product of meiotic DSB but are not due to the non-specifically broken and non-repaired chromosomes, we have examined DSB in cdc1Δ rec12Δ mutant cells. Meiotic DSBs depend on the Rec12 protein, and in its absence meiotic DSBs are not generated (8). The DSBs generated in the cdc1Δ background in the presence of HU are not detected in the rec12Δ background (Fig. 2B), demonstrating that the observed chromosome fragmentation is indeed the product of authentic meiotic recombination. DSBs in cdc1Δ cells in the presence of HU are detected at 3 h after the shift, and their intensities rather increase at later hours as was observed in rad3Δ cells, indicating that the repair of DSBs does not occur or is extremely delayed under this condition (Fig. 2 and supplemental Fig. S1).

DSBs Near the ural1+ Locus Occur at Similar Locations in the cdc1Δ Cells in the Presence of HU—Next we examined the meiotic DNA breaksage of chromosome I. The DSBs were detected by Southern hybridization using a specific probe DNA containing the ural1 gene, which permitted the identification of specific DNA fragments generated by meiotic DSB (8). In the wild-type cells, the specific DNA bands are detected at 3–4 h after the shift, consistent with the results of the pulsed-field gel electrophoresis (Figs. 1B and 3A). In the presence of HU, these bands are not detected. In cdc1Δ cells, the DNA bands with a very similar pattern are detected even in the presence of HU. They appear at 3 h after the shift and are detectable even at 7 h (Fig. 3, B and C). The intensities of these bands are greatly reduced in cdc1Δ rec12Δ cells (Fig. 3C), demonstrating that they are the products of meiotic DSBs. Similarly, the DSBs in the presence of HU were restored in rad3Δ cells as well (Fig. 3D). These results indicate that DSB in cdc1Δ and rad3Δ cells in the presence of HU occurs at the positions common to those of normal meiosis and firmly establish that the Rad3–Cds1 pathway is required for suppression of meiotic DSB in the presence of HU.

We also examined whether cdc1Δ can suppress loss of meiotic nuclear divisions during pat1-induced meiosis in the presence of HU. Whereas nearly 80% of the wild-type cells contained only one nucleus in the presence of HU after incubation for 8 h, the cells with two or more nuclei increased up to 60 or 80% in rad3Δ or cdc1Δ cells, respectively. This increase of multinucleated cells was not observed in chk1Δ or mek1Δ cells (supplemental Fig. S2), indicative of partial recovery of nuclear divisions in the cells compromised for the Rad3–Cds1 pathway. This is consistent with the previous observation made in diploid cells (2).

Induction of rec+ Gene Transcription Is Not Affected by HU Treatment—Initiation of meiotic recombination depends on a number of proteins including various rec+ gene products and replication factors (14, 18). These genes are transcriptionally induced during the meiotic process prior to induction of DSB (19). We have examined transcriptional induction of various rec+ genes in the presence of HU in both wild-type and cdc1Δ cells in order to test the possibility that presence of HU affects the transcriptional induction of these genes and that Cds1 may target them for suppression of DSB in the absence of DNA synthesis (Fig. 4). The transcription of rec6, rec7, and rec12, which is required for meiotic DNA breakage, is induced at 2 h after the shift and peaks at 3 h in the wild-type cells. It is induced at similar or slightly earlier timing in cdc1Δ cells. The transcripts disappear by 5 h after the shift (Fig. 4A). In the presence of HU, the induction of rec+ genes occurs with similar timing and to a similar level, but their transcripts are maintained at a significant level until 8 h after the shift (Fig. 4B). This may indicate that shut off of the rec+ gene transcripts requires completion of DNA replication, recombination, or meiosis. In cdc1Δ cells in the presence of HU, induction occurred at timing similar to that of the wild-type cells, and the transcripts returned to the basal level in a time course similar to that of the wild-type in the absence of HU. This may suggest that Cds1 may inhibit the shut off of the induced rec+ genes in the presence of HU. These results show that the absence of DNA synthesis

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does not affect the induction timing and the level of rec^{+} genes, strongly suggesting that expression of at least some rec^{+} genes is not the target of HU-mediated inhibition of meiotic recombination.

HU-induced Inhibition of Transcription of mei4{sup}^{+} and cdc25{sup}^{+} Is Abrogated in cds1 Mutant—mei4{sup}^{+} is a meiosis-specific transcription factor required for induction of genes involved in sporulation during the later stages of meiosis (13, 20, 21). It was recently reported that mei4{sup}^{+} is required for DSB formation (22). Iino et al. (13) reported that transcriptional induction of mei4{sup}^{+} is impaired by HU treatment, suggesting that mei4{sup}^{+} transcription depends on DNA replication. Consistent with this report, the mei4{sup}^{+} transcript, which is sharply induced at 3–5 h after the shift, is very poorly induced in the presence of HU in the wild-type cells.
In *cds1*/*H9004* mutant cells, the *mei4*/*H11001* transcript is induced with the timing almost identical to the wild-type cells. In the presence of HU, the induction is delayed by 1 h, but the transcript reaches to a level comparable with that in the absence of HU. Similarly, the transcription of *spo6*/*H11001*, *mde2*/*H11001*, and *mde3*/*H11001*, known targets of Mei4 protein (20, 21) is induced in *cds1*/*H9004* cells (albeit with a 1-h delay) but not in the wild-type cells in the presence of HU (Fig. 4, B and C). These results indicate that Cds1 inhibits the *mei4*/*H11001* transcription in response to HU treatment during meiosis in fission yeast.

Inhibition of DNA replication by HU causes persistent inhibitory phosphorylation of tyrosine 15 of Cdc2 kinase, and this may be at least partially responsible for inhibition of meiosis I and II by HU (2). The tyrosine 15 phosphorylation is a target of Rad3-Cds1 checkpoint regulation during meiosis, because it is dephosphorylated in *rad3*/*H9004* and *cds1*/*H9004* cells treated with HU. However, it is not known how Rad3-Cds1 suppresses the tyrosine 15 dephosphorylation in response to inhibition of DNA replication during meiosis. Iino et al. (13) reported that the transcription of *cdc25*/*H11001*, encoding the phosphatase responsible for dephosphorylation of Cdc2 tyrosine 15, thus for activation of Cdc2 kinase, is suppressed by HU treatment during *pat1*-induced meiosis. Indeed, the *cdc25*/*H11001* transcription, which is normally induced at 4–5 h after *Pat1* inactivation, is not induced in the presence of HU (Fig. 4). We

\[ \text{Equation} \]

\[ \text{Figure 3. Measurement of meiosis-specific DNA breakages at specific sites on chromosome I.} \]

Wild-type (WT, JZ767) (A), *cds1Δ* (KO194) (B), *cds1Δ rec12Δ* (KO194), *cds1Δ rec12Δ* (KO273) (C), and *rad3Δ* (KO350) (D) cells were induced to enter meiosis by temperature shift to 32 °C with or without 24 mM HU. Chromosomal DNA was run under a different condition on pulsed-field gel electrophoresis and was stained with ethidium bromide (left panels) as described under “Experimental Procedures” and was transferred to nylon membrane. Southern blot analysis was conducted with a *ura1* gene probe located ~0.75 Mb from the left end of the chromosome I (right panels). *M* indicates *S. cerevisiae* chromosome DNA markers (BioWhittaker Molecular Applications). Arrowheads (a, b, and c) indicate three major fragments that are generated during meiotic DSB and can be detected by this probe. E, the intensities of the three bands (a, b, and c) in panels A–D were quantified, and the relative values at the indicated times are presented.
therefore examined whether this inhibition is abrogated by the cds1 mutation. In cds1Δ cells, the cdc25 transcription is induced in the presence of HU to a level comparable with that in the absence of HU. Thus, it is an intriguing possibility that the Rad3-Cds1 checkpoint pathway may target the transcription of mei4 and cdc25 for suppression, which may concomitantly inhibit meiotic DSB formation and meiotic cell division, respectively, in response to inhibition of DNA replication.

**DISCUSSION**

Meiotic recombination is initiated by DSBs generated by Rec12 protein (8) and is followed by repair of the generated ends through homologous recombination. It is known to be strictly coupled to premeiotic DNA synthesis in *Saccharomyces cerevisiae* (3–5). Our studies indicate that the Rad3-Cds1 checkpoint pathway regulates the coordination of meiS with the initiation of meiotic recombination, namely introduction of DSBs.

**FIGURE 4.** Northern analyses of transcripts of various genes in HU-treated wild-type and cds1Δ cells. A and B, wild-type (WT, JZ767) and cds1Δ (KO194) cells were induced to enter meiosis in the absence (A) or presence (B) of 24 mM HU. At each time point, total RNA was extracted and analyzed by Northern analysis with probes for the genes indicated. C, quantification of the Northern analyses. The band intensities of the Northern data in panels A and B were quantified, and the values relative to that of the time point showing the maximum band intensity in each strain (which is taken as 100) are presented for each probe.
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FIGURE 5. A model for Rad3-Cds1-mediated checkpoint regulation during meiosis.
A model for meiotic checkpoint regulation involving Rad3-Cds1 kinase is presented. HU activates Rad3-Cds1, and Cds1 may inhibit transcription of mei4*. Mei4 protein is required for expression of downstream target proteins, including spo11*, mde2*, and cdc25*. Cds1 may regulate the transcription of cdc25* in a more direct manner as well. X represents a hypothetical factor such as mde2* that may regulate DSB formation and whose expression may be regulated by mei4*.

It was previously reported that Rad3 regulates coupling of meiS with meiotic cell division (1, 2). Inhibition of meiS by HU inhibits meiosis I and II, and this inhibition is partially circumvented by rad3Δ or cds1Δ through decreased phosphorylation of tyrosine 15 of Cdc2 (2). Another checkpoint involving Mek1 kinase, related to Cds1 (6, 7), operates to coordinate recombination and cell division. Our data indicate that the Rad3-Cds1 pathway is also involved in suppression of formation of DSBs in the absence of premitotic DNA replication. It is interesting to note that the following repair of the generated DNA ends does not take place or is at least extremely delayed in HU-treated rad3Δ or cds1Δ cells (supplemental Fig. S1), suggesting that this step may be regulated by an as yet unknown checkpoint pathway. While we were preparing this report, Tonami et al. (9) reported that suppression of meiotic DSB formation in the presence of HU during fission yeast meiosis is relieved in rad3Δ, cde1Δ, and rad26Δ cells as well as in checkpoint clamp-clamp loader mutant (rad1Δ, rad9Δ, hus1Δ, rad73Δ) cells.

The next question concerns the nature of the critical target of Cds1 in suppression of DSB formation in HU-treated meiotic cells. We have examined transcriptional induction of various rec* genes and other factors. Our data indicate that induction of at least some rec* genes whose products are required for DSB formation (18, 22) is normal in both wild-type and cds1Δ cells treated with HU (Fig. 4). On the other hand, the level of induction of mei4*, encoding a meiosis-specific transcription factor required for expression of genes involved in later stage of meiosis (20, 21), is substantially reduced in wild-type cells treated with HU, as was reported previously (13). Consistent with this, the transcription of the target genes of Mei4 protein was also reduced.

Interestingly, transcripts of mei4* and its target genes were restored to the control level in cds1Δ cells in the presence of HU. Furthermore, cdc25* induction, which is also inhibited by HU in the wild-type cells, is restored in cds1Δ cells in the presence of HU. Because mei4* is required for DSB formation (22) and Cdc25 is an activator of cell division, these results lead us to propose an intriguing model that transcription of mei4* and cdc25* may be a target of the Rad3-Cds1 checkpoint during meiosis and that suppression of these genes in the presence of HU would concomitantly inhibit both DSB formation and meiotic cell divisions as well as sporulation (Fig. 5). mde2*, a target of Mei4, was recently reported to be required for meiotic DSB formation (23). The transcription of mde2* is under the regulation of Cds1 (Fig. 4) and thus may be one of the critical targets of Mei4 during this meiotic checkpoint regulation (X in Fig. 5). It is known that chromatin remodeling is required for DSB formation at a recombination hot spot (24–26). We observed significant reduction of histone acetylation at ade6-M26 after HU treatment.3 Thus, Cds1 protein activated by HU may inhibit the reorganization of the chromatin favorable for DSB formation and expression of the putative target genes of Rad3-Cds1 checkpoint. In cds1Δ cells, chromatin structures may be maintained in a state permissive for DSB and for gene expression even in the presence of HU. Cds1 may inhibit the expression of mei4* and cdc25* independently. Alternatively, down-regulation of cdc25* expression may be due to decreased expression of mei4*, because the level of cdc25* transcript significantly decreases during meiosis in mei4Δ cells (13). At present, however, we cannot entirely rule out the possibility that loss of expression of these genes by HU treatment may somehow result from the absence of meiotic recombination and that Rad3-Cds1 may inhibit DSB formation through some other target(s). We are currently conducting experiments to test the model.

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