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The Signal from the Initiation of Meiotic Recombination to the First Division of Meiosis

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Two of the unique events that occur in meiosis are high levels of genetic recombination and the reductional division. Our previous work demonstrated that the *REC102*, *REC104*, *REC114*, and *RAD50* genes, required to initiate meiotic recombination in *Saccharomyces cerevisiae*, are needed for the proper timing of the first meiotic (MI) division. If these genes are absent, the MI division actually begins at an earlier time. This paper demonstrates that the meiotic recombination genes *MER2/REC107*, *SPO11*, and *MRE2* and the synaptonemal complex genes *HOP1* and *RED1* are also required for the normal delay of the MI division. A *rec103/ski8* mutant starts the MI division at the same time as in wild-type cells. Our data indicate no obvious correlation between the timing of premeiotic S phase and the timing of the first division in Rec^{-} mutants. Cells with *rec102* or *rec104* mutations form MI spindles before wild-type cells, suggesting that the initiation signal acts prior to spindle formation. Neither *RAD9* nor *RAD24* is needed to transduce the signal, which delays the first division. The timing of the MI division in *RAD24* mutants indicates that the pachytene checkpoint is not active in Rec^{+} cells and suggests that the coordination between recombination and the MI division in wild-type cells may occur primarily due to the initiation signal. Finally, at least one of the targets of the recombination initiation signal is the *NDT80* gene, a transcriptional regulator of middle meiotic gene expression required for the first division.

Chromosomes passing through meiosis undergo a specific sequence of events that does not occur in mitotic cells. These events include an S phase that may well be unique to meiosis, high levels of genetic recombination, formation of the synaptonemal complex (SC), a reductional division, and an equational division that is not immediately preceded by an S phase (27, 33, 41). It has become clear that these meiotic events are coordinated, not only through a sophisticated temporal regulation of gene expression (5, 23, 36), but also by the existence of meiotic checkpoints that assess whether events have occurred properly (e.g., reference 42). A checkpoint in prophase I has been studied in detail and has been referred to variously as the pachytene checkpoint and the recombination checkpoint (18, 29).

At least two types of defects can be recognized by the pachytene checkpoint: defects caused by mutations in some late recombination genes such as *dmc1* (i.e., "late" because they act after the formation of double strand breaks [DSBs]) and defects caused by mutations in some SC genes such as *zip1*. In such mutant strains, cells arrest in late prophase and do not undergo the first meiotic (MI) division. The ability to establish the checkpoint depends on the presence of part of the mitotic DNA damage checkpoint system; *RAD24* and *RAD17* are required for the arrest (29, 42). In contrast, *RAD9* (which is required for the mitotic checkpoint) does not play a role in the pachytene checkpoint. Evidence suggests that the long single-strand tails that exist in *dmc1* mutants (3) are recognized by the pachytene checkpoint. It has been suggested that the pachytene checkpoint might act in normal Rec^{+} cells to coordinate recombination and the MI division because exonuclease digestion after DSB formation normally results in single-stranded DNA (ssDNA) during recombination (29). Of course, in Rec^{+} cells, the ssDNA tails are shorter and not persistent (49), consistent with the idea that the normal ssDNA could cause a delay rather than an arrest of the first division.

In earlier work, we found that the genes that act in the initiation of meiotic recombination to form DSBs also play a role in coordinating the timing between recombination and the first division (15, 21). Ten genes are required to form DSBs: *REC102*, *REC103/SKI8*, *REC104*, *MER2/REC107*, *REC114*, *MEI4*, *SPO11*, *RAD50*, *XRS2*, and *MRE11* (25). There are two genes (*MRE2* and *MER1*) required to splice the intron present in *MER2/REC107*; mutations in these two genes confer phenotypes similar to those of initiation mutants. All of these genes have been classified as early recombination functions, because they are required to make DSBs and are rescued by the presence of a *spo13* mutation (e.g., see reference 30). The *SPO11* gene has been proposed to code for the protein that is directly involved with making DSBs; support for this hypothesis takes two forms. First, *SPO11* has homology to type II topoisomerases involved in making DSBs as they pass one strand of DNA through the other (2). Second, Spo11p is found covalently attached to the ends of DSBs in certain mutants, which can make breaks but not process them (26). However, it is absolutely clear that the other nine genes are all required to make breaks. No breaks are observed when any of the genes are deleted (25), even if the Spo11p is tethered to the DNA by the binding domain of another protein (38).

The presence of all of the recombination initiation genes

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results in the normal timing of the first division. Our previous work discovered that the MI division begins at earlier times in cells lacking some of the recombination initiation genes (15, 21). It was initially tempting to attribute the delay of the MI division in Rec$^+$ wild-type (WT) cells to the formation of DSBs, since even a single DSB in a mitotic cell can result in cell arrest (1, 12). However, *mei4* mutants started the first division at the same time as Rec$^+$ cells, yet no DSBs were observed in such cells (15, 21). This suggested that neither DSB formation nor recombination, per se, was the signal that resulted in the normal delay of the first division observed in Rec$^+$ cells. We discovered that *rec104* and *rec114* cells displayed an early MI division starting about an hour before WT cells. Mutants containing either *rad50* or *rec102* exhibited a very early first division, starting about 2 h earlier than WT cells. Recent work by Kee and Keeney (24) indicates that *spo11* mutants also start the MI division earlier than WT cells. When we constructed double mutants containing a "very early" mutation and an "early" mutation (e.g., *rec102 rec104*), the very early mutation was always epistatic to the early mutation. Because the recombination initiation mutations confer an earlier first division, it is important to realize that the delayed timing of MI observed in WT cells is a normal feature of meiosis.

Another group of genes is involved early in meiotic recombination; this group includes the genes coding for proteins involved in the formation of the SC. Two genes we examine in this paper are *RED1* and *HOP1*. These two genes play a role in the formation of axial elements and are components of the mature SC (20, 47). Unlike mutations in the 10 recombination initiation genes required for DSBs, null mutations in *hop1* or *red1* do not completely abolish meiotic recombination or DSBs. Typically about 10% of the normal meiotic levels of recombination can be detected in *red1* or *hop1* mutants (19, 32, 40).

In this paper, we investigate what other recombination initiation functions are required for the normal signal to delay the first division and if components of the SC are required. Recently, Kleckner and colleagues (4) have published data that indicate that *spo11* has a shorter premeiotic S phase than Rec$^+$ cells; they suggested that this might be the reason that *spo11* cells enter the MI division earlier. We address this proposal by examining the S phase in several recombination initiation mutants. Experiments are also presented that determine whether the initiation signal acts before or after MI spindle formation. Data are presented on the role of *RAD24* and *RAD9* in the initiation signal, and the results bear on the role of the pachytene checkpoint in WT Rec$^+$ cells. Finally, experiments are presented that ask about the target of the recombination initiation signal that delays the MI division.

MATERIALS AND METHODS

Yeast strains and mutations. All strains used in this paper are isogenic and are derivatives of the homothallic diploid K65-3D (15), ultimately derived from S288C. Our strains display classical kinetics of meiosis (11): slower than SK strains and faster than BR strains. K65-3D is homozygous for the following mutations: *HO*, *lys2-1*, *tyr1-1*, *his7-2*, *can1^r*, *ura3-13*, *ade5*, *met13-d*, *trp5-2*, *leu1-12*, and *ade2-1*. All of the recombination and checkpoint mutations used were null deletion mutations. Some mutations have been described previously: *red1Δ::URA3* (50), *hop1Δ::URA3* (19), *mei4Δ::URA3* (15), *rec104-Δ1* (16), *rec102Δ-2::LYS2* (15), and *rad50Δ::URA3* (21). All strains containing the G418^R gene were precise deletions of the coding region and were obtained from the Research Genetics deletion collection. The deletions are described by the *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). All deletion and deletion/insertion mutations were tested by both genetic analysis and Southern blottting.

Media, growth, and sporulation conditions for sporulation experiments. The media, growth, and sporulation conditions have been described previously (15). For each experiment, all cultures were grown in the same batch of medium and were treated identically. In every experiment, we examined sporulation of one culture of a WT strain (K65-3D), one culture of a *rec104* strain, and two cultures of the mutant being examined. The WT and *rec104* strains were included as normal and early timing controls, respectively. All timing experiments were repeated at least twice. Time points were taken every half hour through the period when the MI division initiates, and at least 1,000 cells were counted for each point. We note, as previously reported (15), that the exact timing of events in sporulation can vary slightly from one experiment to another, but the relative timing never varies (e.g., *rec104* mutants always begin the MI division earlier than *REC104* cells).

Microscopy. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI), and cells were examined with a fluorescent microscope as described previously (15), except that cells were suspended in mounting medium by using 65% glycerol. For time points of ≤3 h, at least 400 cells were counted; for all subsequent time points, at least 1,000 cells were counted. Mononucleate cells include those that have not yet undergone the first division. Binucleate cells consist of cells with two distinguishable DAPI-staining nuclei. (Binucleate cells that represent events in mitotic cells are easily distinguishable because the two nuclei are located at the bud/cell junction; such cells aren't counted in the data. The number of mitotic cells with two nuclei falls to <0.5% by 2 h in sporulation medium.) The final degree of sporulation for WT K65-3D cells in these experiments ranged from 65 to 75% mature asci. Meiosis-specific Rec$^-$ mutants displayed sporulation frequencies ranging from about 20 to 30%. Sporulation frequencies of *rad50* and *rec103/ski8* mutants ranged from 5 to 15%.

Tubulin staining used standard protocols (17, 39). Briefly, cells obtained from time points were immediately fixed in 4% formaldehyde for 24 h, and about 10^7 cells from each time point were permeabilized by incubation in 60-μg/μl zymolase, 2% glusulase, and 0.8% β-mercaptoethanol for 40 min at 37°C in 40 mM phosphate buffer with 1.2 M sorbitol and 0.5 mM $MgCl_2$. Cells were then incubated in 75% ethanol for 5 min followed by an hour of incubation at room temperature with a 1:500 dilution of YOL1/34 antitubulin rat monoclonal antibody (no. MCA78S; Serotec) in a mixture of 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid; pH 6.9], 5 mM EGTA, 5 mM $MgCl_2$, and 2% bovine serum albumin. The secondary antibody used was Alexa 488-conjugated goat anti-rat immunoglobulin G (no. A-11006; Molecular Probes) at a 1:1,000 dilution. Cells were resuspended in a DAPI solution consisting of 1-μg/ml DAPI in sodium bicarbonate buffer (pH 9.0) with 65% glycerol. Stained cells were examined with a Leitz Laborlux 12 microscope equipped with fluorescence filters and a Pixera Pro 150ES digital camera. Viewfinder 3.0, Studio 3.0, and Adobe Photoshop 7 software were used to obtain the images in Fig. 5. For time points of <4 h, at least 500 cells were counted; for time points of ≥4 h, at least 1,000 cells were counted. Both short and long MI spindles were counted as soon as they became detectable. Mitotic spindles and MII spindles were easily distinguished from MI spindles by their morphology.

Northern analysis. Cell pellets obtained from meiotic time point experiments were frozen at −75°C until RNAs from these cells were isolated as previously described (31). Northern (RNA) blot analysis was performed as described previously (6). DNA probes were made by PCR and encompassed the coding regions and approximately 100 bp upstream and downstream. Probes were labeled with [α-^{32}P]dATP by using the Invitrogen Life Technologies Random Primers DNA labeling system. Northern blots were analyzed with a Molecular Dynamics PhosphorImager (model 445SI) and ImageQuant software.

Analysis of premeiotic DNA synthesis by flow cytometry. For the experiments analyzing premeiotic DNA synthesis, cells were grown and sporulated as described above with the following differences: Diploid cells were grown to saturation in YPD (1% yeast extract, 2% peptone, 2% glucose), diluted in YPA (1% yeast extract, 2% peptone, 1% potassium acetate), and grown to 4×10^7 to 6×10^7 cells/ml to reduce the number of mitotic cells that were 4C upon entering sporulation medium. Cells were then spun down, washed, and resuspended in sporulation medium as described above. Aliquots (0.5 ml) of cells were collected at each time point, pelleted, resuspended in 70% ethanol, and stored at 4°C. Prior to staining, cells were treated as follows. (i) The cells were twice pelleted and resuspended in 50 mM sodium citrate (pH 7.5) at room temperature for at least 30 min. (ii) They were then pelleted and resuspended in 50 mM sodium citrate containing 0.25-μg/ml RNase A and (iii) incubated at 37°C for 16 to 20 h. (iv) Proteinase K was added to a concentration of 20 μg/ml. (v) Finally, these

samples were incubated at 50°C for 3 h. Propidium iodide was then added to a final concentration of 5 μg/ml. Cells were stored at 4°C in the dark. On the day of fluorescence-activated cell sorting analysis, cells were sonicated for 20 s at 40% power with an Artek 150 Sonic Dismembrator. Samples were analyzed on a Becton Dickinson FACSCalibur using CellQuest software. For each sample, 10^5 events were collected. Strains were sporulated and analyzed in three independent experiments to determine the average values, except for the *rec114* strain, for which experiments were done twice.

Once events were collected, cell cycle analysis was performed with FlowJo software. The Dean-Jett-Fox model (7, 10, 13), incorporated into the cell cycle analysis option of FlowJo, was used to determine the fraction of cells in the 2C, S, and 4C phases at any particular time. Once the fraction of cells in S phase was determined, the method of Cha et al. (4) was employed to determine the length of S phase. Using the length of S phase, we calculated a cumulative curve (4) to determine the time at which 50% of cells had entered S phase. We also calculated the time when 50% of cells had entered S phase by plotting the fraction of 2C cells versus time and determining when 50% had left the 2C peak. This calculation assumes that cells no longer in 2C have entered S phase. This provides an independent measurement of S-phase entry that does not depend on the calculation of S-phase length to create a cumulative curve.

RESULTS

The recombination initiation signal for the delay of the first division. Throughout this paper, we define cells that have segregated their chromosomes into two separated and distinguishable nuclei as having undergone the MI division. Recombination initiation genes previously shown to be required for the normal delay of the first division include *REC102*, *REC104*, *REC114*, and *RAD50*. Kee and Keeney (24) recently demonstrated that *spo11* mutants also begin the first division earlier. The data in Fig. 1 indicate that *MER2/REC107* and *MRE2* are also required for the WT delay; mutants in these genes have an earlier reductional division. The *spo11* mutant in our strain background acted in the same manner as observed by Kee and Keeney in the SK1 background (24); our independent data in a different strain background support their conclusion that normal timing of the first division requires *SPO11*. A strain lacking *REC103/SKI8* began the MI division at the normal time; *REC103/SKI8* is apparently not needed for the recombination initiation signal to the first division.

The timing of the first division in cells lacking *SPO11* or *MER2/REC107* was indistinguishable from the that of the early division that occurs in the *rec104* control. We note that the timing of the first division in *mre2* cells, which lack an mRNA processing function required for *MER2/REC107* (35), also occurred at an earlier time that was indistinguishable from that of *rec104* cells (See Discussion).

Double mutant analysis of meiotic recombination initiation mutants. In our previous work (15, 21), the recombination initiation mutants displayed patterns of epistasis consistent with their acting in a linear pathway with respect to timing of the first division. To determine whether the mutations examined in Fig. 1 above could also be placed in the same formal pathway, we examined a number of double mutants. As expected from our earlier results, *spo11* was epistatic to *mei4* (Fig. 2A); the double mutant displayed the early division phenotype. Likewise, the *mer2/rec107* and the *mre2* mutations were epistatic to *mei4* (Fig. 2B and C). The double *rec104 mre2* mutant had timing indistinguishable from that of either mutant alone (Fig. 2D). This observation suggests that the *rec104* and *mre2* mutations affect the same process.

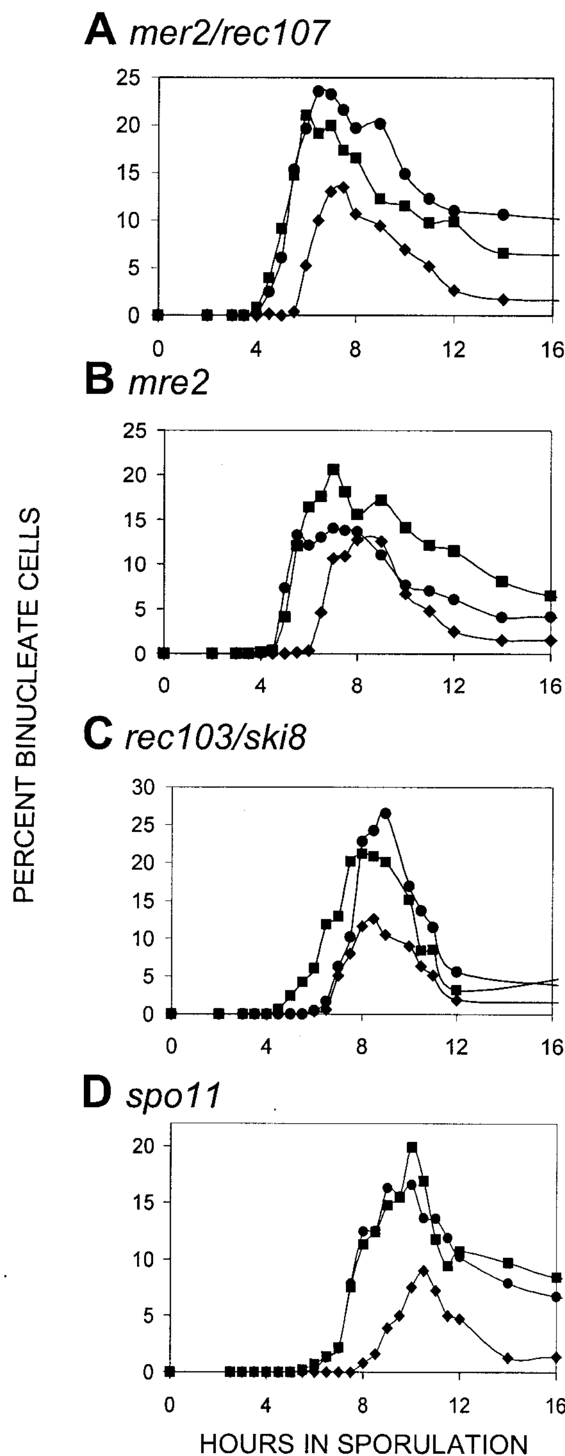


FIG. 1. Timing of the first division in mutants affecting recombination initiation. In all of the graphs, the first division of WT cells is shown as solid diamonds (♦) and is a control for normal timing. The control for early timing of the first division in all panels is a *rec104* strain denoted by solid squares (■). In all of the graphs, the mutant examined is shown as solid circles (●); for each mutant, at least two independent cultures were examined for each experiment and the data point shown is the mean. Another independent experiment was done for each mutant; in each case the repeat gave the same result (data not shown). The type of mutant examined is shown above each panel.

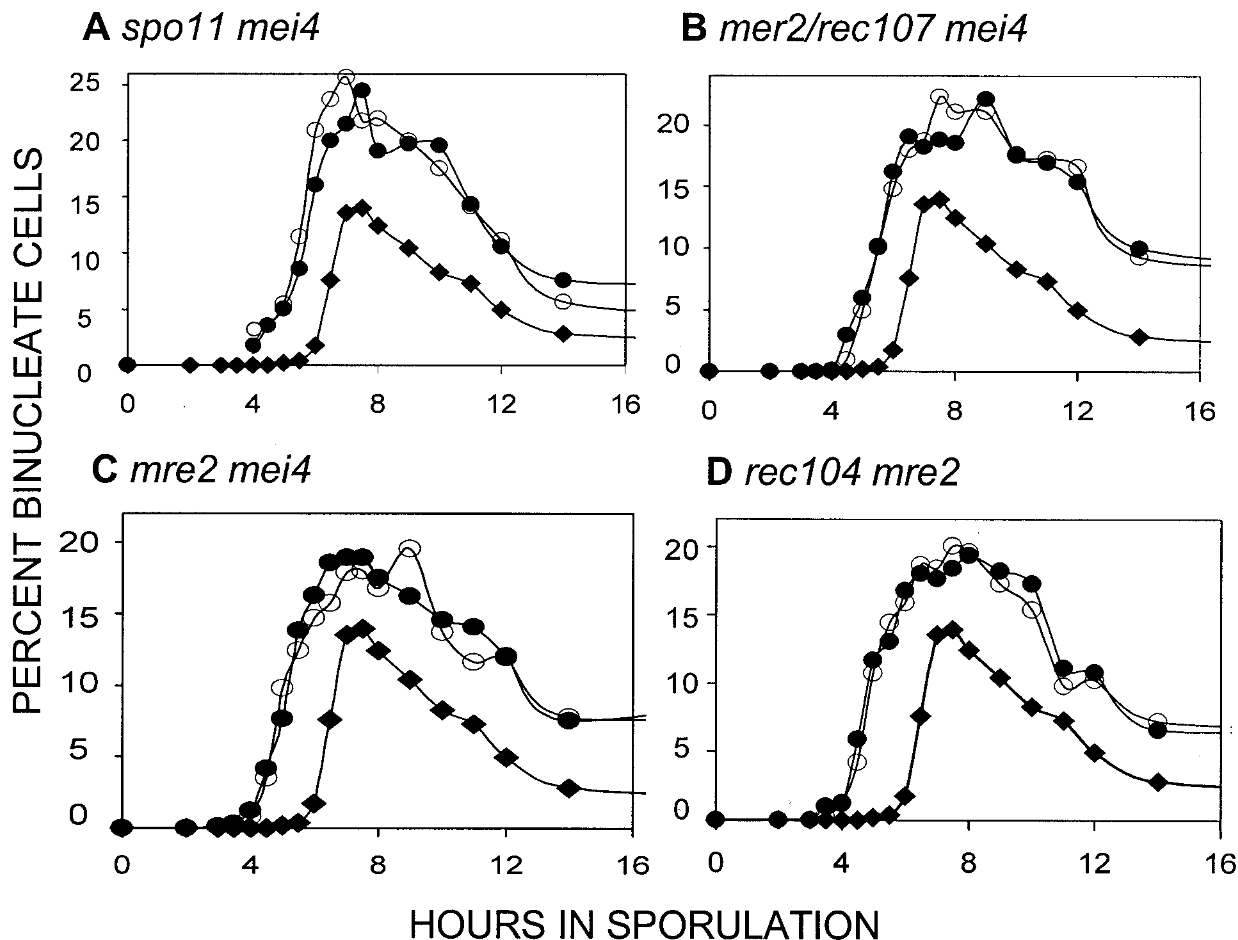


FIG. 2. Timing of the first division in double mutants. The type of double mutant examined is shown above each panel. In each panel, WT cells are denoted by solid diamonds (♦). Two independent cultures of each double mutant are shown and denoted by open and solid circles (○, ●). (A to D) All double mutant cultures and the WT control were done at the same time; the WT curves are identical in each panel.

Are components of the SC required for the initiation signal? In *Saccharomyces cerevisiae*, the axial elements (future lateral elements) of the synaptonemal complex appear to form at a time in recombination when the initiation functions are acting (41). We considered two possibilities for the role of Red1p and Hop1p in the initiation signal. First, if they were part of a recombination initiation complex that signaled for the normal delay of the first division, *hop1* and *red1* mutants should have an earlier MI division. Alternatively, both *red1* and *hop1* mutants display about 10% of the WT level of meiotic recombination (19, 32, 40); this level represents about a 20- to 30-fold induction over the mitotic background. If this level of recombination were sufficient to signal the initiation of recombination, *hop1* and *red1* strains should have normal timing. The data in Fig. 3 indicate that in both *hop1* and *red1* strains, the first division starts at an earlier time indistinguishable from that of the *rec104* control. This indicates that these SC proteins do play a role in the normal signal for delay.

Premeiotic DNA synthesis in meiotic recombination initiation mutants. Recently, Cha et al. (4) examined premeiotic S phase in several meiotic mutants. Of the mutants examined, two were deficient in meiotic recombination initiation (*spo11*Δ and *rec102*Δ). The authors reported that the length of premeiotic S phase is shorter in *spo11*Δ cells (59 min) than in WT cells (77 min) or *rec102*Δ cells (72 min). However, the times at which 50% of the WT, *rec102*Δ, or *spo11*Δ cells entered premeiotic S phase were indistinguishable. The shorter S-phase length of *spo11*Δ mutants suggests a possible reason for the earlier MI division, although the normal length of S in *rec102*Δ cells (4) is not consistent with that idea.

To test this possibility, premeiotic DNA synthesis was examined in the WT and *spo11*, *rec102*, *rec104*, *rec114*, *mei4*, and *rec103/ski8* gene deletion mutants (Fig. 4). Using the methods of Cha et al. (4), curves were plotted for each culture (Fig. 4B), and the length of the S phase was calculated (Table 1). The average of three independent experiments shows that all of the mutants exhibit S-phase lengths of 55 to 59 min (Table 1). None of these values is significantly different from the S-phase length of WT cells or from each other (Table 1). The calculated S-phase lengths for WT cells and *spo11*, *rec102*, *rec104*, and *rec114* mutants (all of which have an early MI division) are indistinguishable. We did not calculate S-phase length for *mei4* or *rec103/ski8* cells, since only one experiment was done for each. However, we do note that there are no obvious differ-

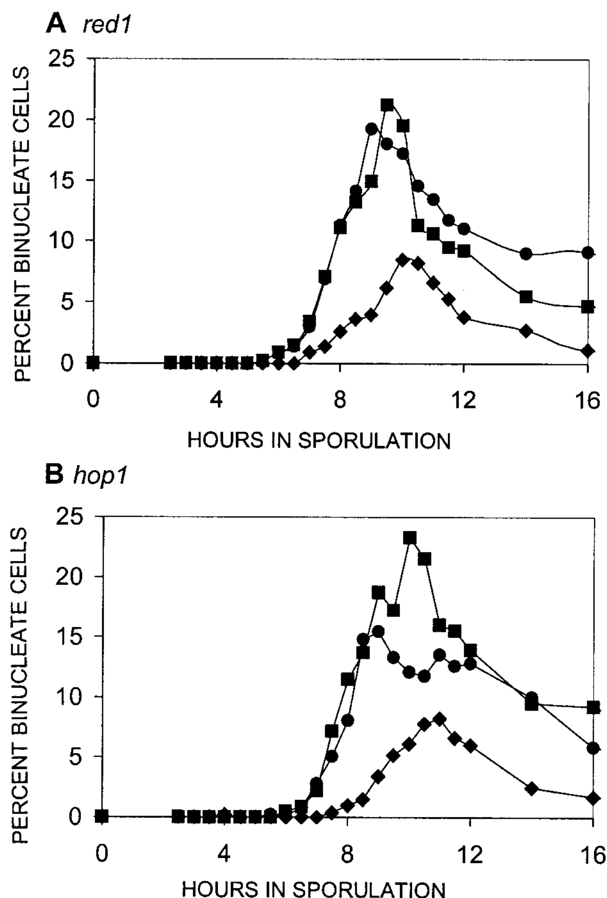


FIG. 3. Timing of the first division in SC mutants. In all of the graphs, the first division of WT cells is shown as solid diamonds (♦) and is a control for normal timing. The control for early timing of the first division in all panels is a *rec104* strain denoted by solid squares (■). In all of the graphs, the mutant examined is shown as solid circles (●). For each mutant, at least two independent cultures were examined for each experiment and the data point shown is the mean. Another independent experiment was done for each mutant. In each case, the repeat gave the same result (data not shown). The mutant examined is shown above each panel.

ences in the histograms (Fig. 4A) between the WT up to the 600-min time point with the *mei4* mutant or up to the 360-min time point with the *ski8/rec103* mutant. Cumulative curves of cells entering S phase were calculated by the methods described by Cha et al. (4) (Fig. 4C). In the WT and all mutants examined, 50% of the cells enter premeiotic S phase at about 213 min after introduction into sporulation medium. There were no significant differences between the Rec$^-$ mutants and the WT cells. Finally, we determined a time of entry into S phase, using a method independent of the calculation of S-phase life span (Fig. 4D). This method shows that, in WT cells and all mutants examined, 50% of the cells enter premeiotic S phase at about 240 min after introduction into sporulation medium. There is no significant difference between any of the mutants examined and WT, suggesting that differences in S-phase length do not explain why Rec$^-$ cells start the MI division earlier.

Meiosis I spindle formation in recombination initiation mutants. Shonn et al. (46) have argued that the reason chromsomes separate at an earlier time in Rec$^-$ initiation mutants is that, without chiasmata to provide tension, chromosomes are pulled apart as soon as the spindle is formed in Rec$^-$ mutants; division in WT Rec$^+$ cells with chiasmata is delayed by the spindle checkpoint system. This idea predicts that MI spindles form at the same time in initiation mutants and WT cells, but that chromosome segregation is delayed in WT cells. To test this, we measured the fraction of cells containing MI spindles versus time in sporulation for WT, *rec102*, and *rec104* cells by using fluorescence microscopy with antitubulin antibodies (Fig. 5 and 6). The data clearly show that MI spindles form earliest in *rec102*, then in *rec104*, and then in WT cells. The fact that spindles form at an earlier time in Rec$^-$ cells is not easily reconciled with the hypothesis of Shonn et al. (46) (see Discussion).

Do known meiotic checkpoint functions transduce the recombination initiation delay? The pachytene checkpoint (e.g., as defined by *dmc1* or *zip1* mutants) results in arrest before the first division (3, 42). Mutations in *RAD24* eliminate the arrest; for example, *rad24 dmc1* cells proceed through the first (and second) division (29, 42). We asked if *RAD24* was required for the recombination initiation signal that normally delays the first division: if it were, *rad24* mutants should display an earlier first division. The data in Fig. 7 indicate that *RAD24* does not play a role in the process. In fact, exactly as observed by Shinohara et al. (45), we find that *rad24* mutants begin the first division at a later time. The timing of *rad24* makes the role of pachytene checkpoint in WT cells problematic (see Discussion). Although both *RAD9* and *RAD24* are needed for proper checkpoint activity in the DNA damage pathway in mitosis (8), the *RAD9* gene does not play a role in the meiotic pachytene checkpoint (29). The *RAD9* gene is also not required for the recombination initiation delay signal (Fig. 7B); the timing of the first division is indistinguishable from that in WT cells. The *rec104* mutation is epistatic to both *rad24* (Fig. 7C) and *rad9* (Fig. 7D); we observe no synergistic effects in the double mutant. The data indicate that neither *RAD9* nor *RAD24* is required to transduce the initiation signal that delays the MI division.

The target of the signal for recombination initiation. A candidate for the target of the recombination initiation signal that delays the first division is the *NDT80* gene. This gene codes for a positive regulator of the middle meiotic genes, and *ndt80* mutants arrest before the first division (5, 18, 51). We therefore examined the expression of *NDT80* in the WT and *rec104* and *rec102* mutants (Fig. 8). Expression of the *SPS4* gene was used as a reporter of total *NDT80* activity, since evidence indicates that Ndt80p is also posttransciptionally regulated (36, 51). *NDT80* transcription begins earlier in both *rec102* and *rec104* mutants than in WT cells, although our current data do not distinguish between the two Rec$^-$ mutants. *SPS4* transcription, however, begins earlier and reaches higher levels in *rec102* than in *rec104* (e.g., compare *SPS4* expression at 5 and 6 h of sporulation in Fig. 8A and B), consistent with the earlier start of the MI division. Both Rec$^-$ mutants clearly express *SPS4* prior to the time it is expressed in WT cells. The data indicate that *NDT80* is a target of the recombination initiation signal.

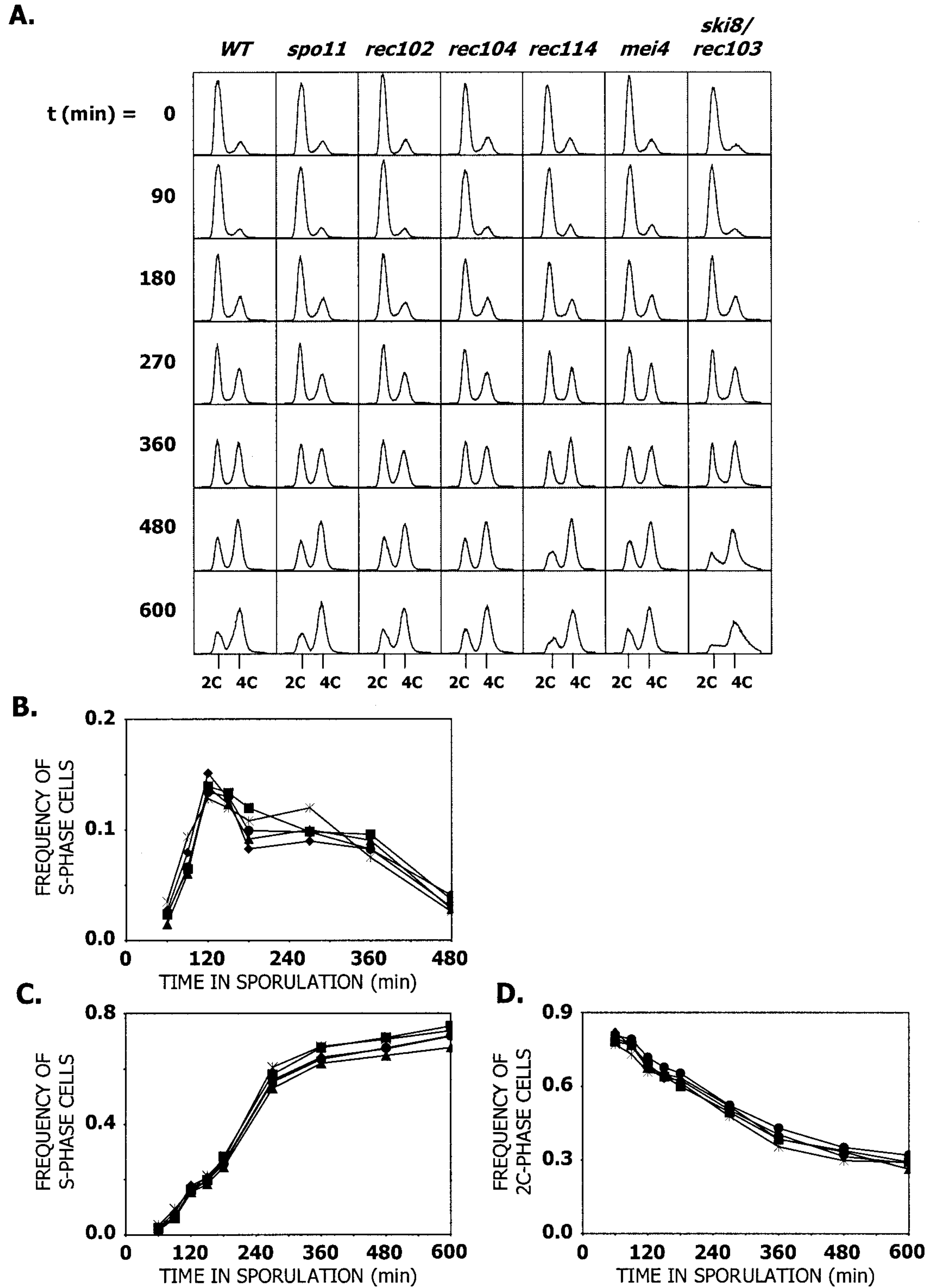


FIG. 4. Analysis of premeiotic DNA synthesis by flow cytometry. (A) Representative histograms showing DNA content in cells proceeding through meiosis. The relevant genotype of each strain is designated above, minutes in sporulation are designated beside and 2C and 4C DNA contents are designated below histograms. (B) Noncumulative curve of cells proceeding through premeiotic S phase. (C) Cumulative curve of cells proceeding through premeiotic S phase. The curve was calculated by the method of Cha et al. (4). (D) Cumulative curve of cells exiting 2C phase (entering S phase). The curve was calculated by using the cell cycle analysis tool in the FlowJo software package. WT, ◆; *spo11*, ▲; *rec102*, ●; *rec104*, ■; *rec114*, *. For all graphs, the average values for three independent experiments are shown for all strains except the *rec114* strain (which had only two independent experiments). The average sporulation frequencies for the experiments were as follows: WT = 74%, *spo11* = 25%, *rec102* = 31%, *rec104* = 20%, and *rec114* = 25%.

TABLE 1. Premeiotic S-phase length and time of entry into S phase of meiotic recombination initiation mutants[a]

Strain type	S-phase length (min)[b]	Time (min) of entry into S phase[c]	Time (min) of exit from 2C phase[d]
WT	55.9 ± 1.3	214 ± 4	234 ± 14
spo11	58.0 ± 5.0	212 ± 7	258 ± 18
rec102	58.1 ± 3.2	215 ± 6	249 ± 25
rec104	58.5 ± 4.5	213 ± 6	232 ± 28
rec114	56.9 ± 0.9	213 ± 9	236 ± 8

[a] All values are the average of three independent sporulation experiments, except for the *rec114* strain (two independent experiments). None of the values is significantly different from WT, nor are they significantly different from each other (as examined by Student's *t* test).
[b] S-phase length calculated by the method of Cha et al. (4).
[c] Time in which 50% of active cells have entered S phase calculated by the method of Cha et al. (4) (Fig. 4C).
[d] Time in which 50% of active cells have exited 2C phase (and therefore entered S phase) as calculated with the cell cycle analysis tool in the FlowJo software package (Fig. 4D).

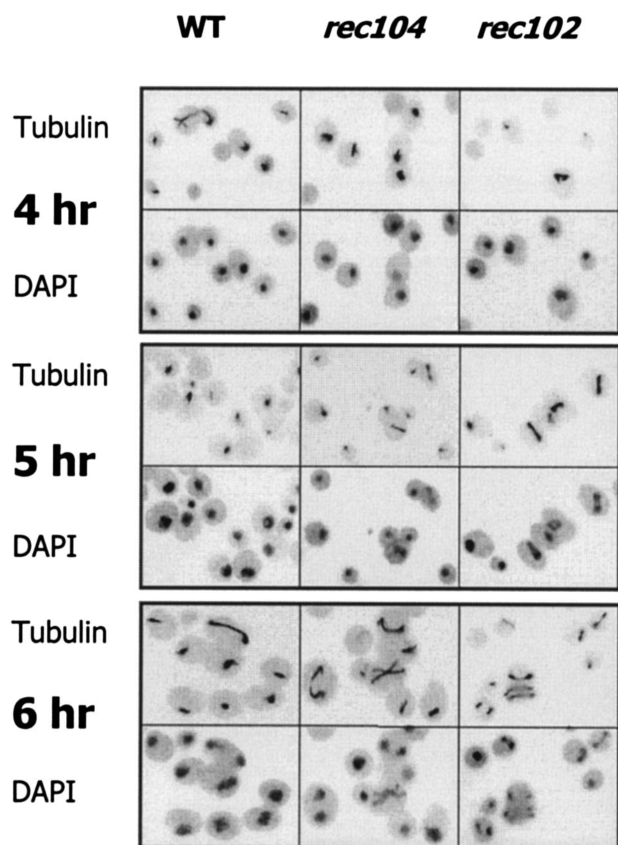
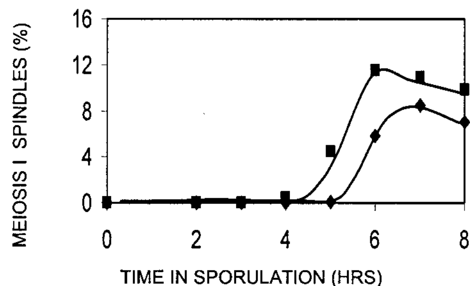
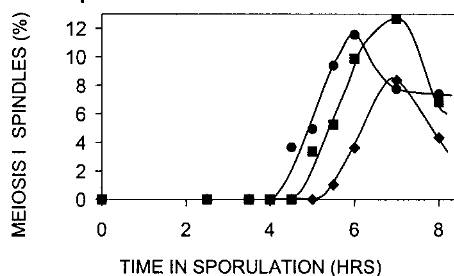


FIG. 5. Meiosis I spindles in WT cells and Rec$^-$ mutants. Fields illustrating the presence of MI spindles are shown at illustrative time points in WT, *rec104*, and *rec102* cells. For each time point, the top figure is a picture of the immunofluoresence observed due to antitubulin antibody. The bottom figure is a picture of the nucleus as observed by DAPI fluorescence.





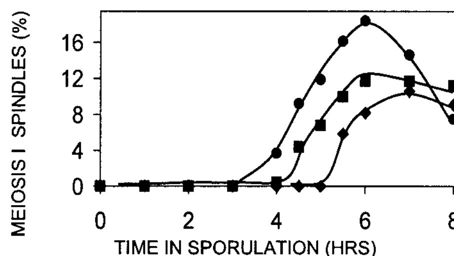


FIG. 6. The timing of MI spindle formation in WT cells and Rec$^-$ mutants. Three independent experiments were performed on different days to determine the time of MI spindle formation in WT cells and *rec104* and *rec102* mutant strains. In each experiment, DAPI staining verified that the timing of the first division was earlier in the Rec$^-$ mutants than in the WT cells (data not shown).

DISCUSSION

For complex biological processes to occur properly, cells must ensure that events happen at the right time and in the correct order. During meiosis, chromosomes undergo premeiotic DNA synthesis, recombination and synapsis, the reductional division, the equational division, and packaging into gametes or spores. Given the complexity of these events, it is not surprising either that there is communication between them or that a number of checkpoint systems exist in *S. cerevisiae* that arrest cells in the progression through meiosis if the preceding step has occurred improperly. For example, a checkpoint for proper premeiotic DNA synthesis (48) arrests cells prior to recombination. One of the best-studied checkpoints occurs in pachytene (42), where defects in some of the steps of recombination and synapsis (e.g., *dmc1*, *zip1*, and *hop2*) result in arrest. This checkpoint is mediated by components of the mitotic DNA damage checkpoint system and requires *RAD24*, *RAD17*, *DDC1*, etc. Unlike the mitotic DNA damage check-

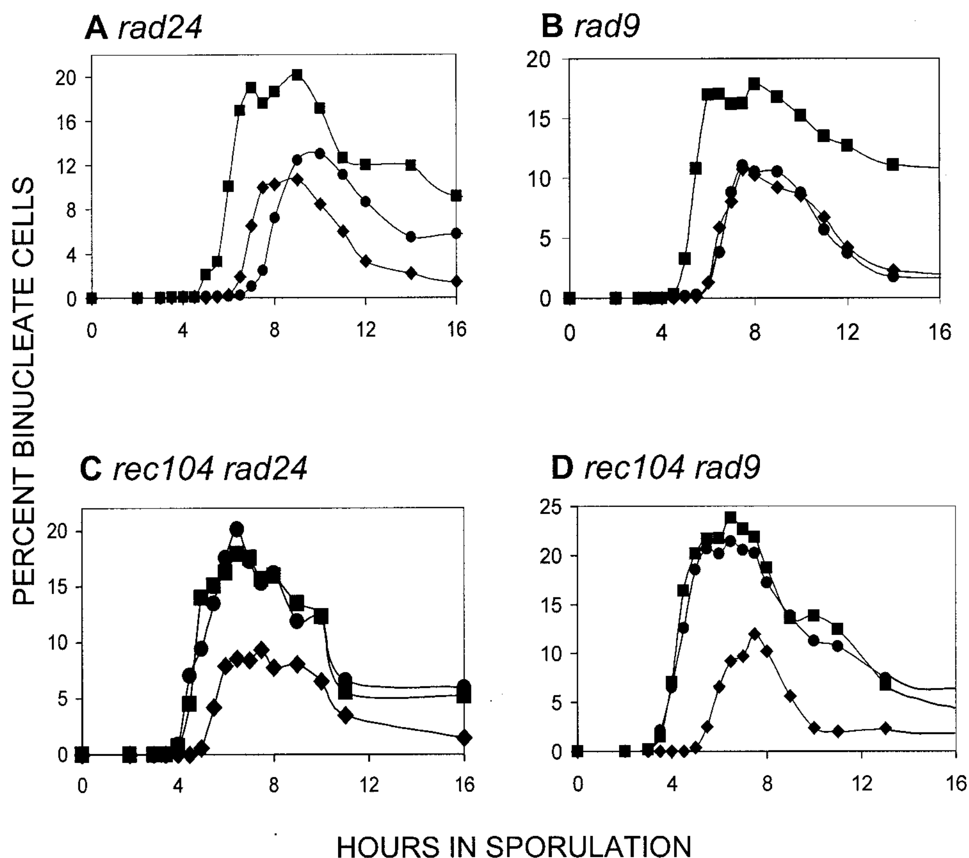


FIG. 7. Timing of the first division in checkpoint mutants. In all of the graphs, the first division of WT cells is shown as solid diamonds (◆). The control for early timing of the first division in all panels is a *rec104* strain denoted by solid squares (■). In panels A and B, the mutant in question is shown by solid circles (●); in panels C and D, the solid circles refer to the double mutant. The type of mutant examined is shown above each panel. For each mutant, at least two independent cultures were examined for each experiment, and the data point shown represents the mean. Another independent experiment was done for each mutant; in each case, the repeat gave the same result (data not shown).

point, the pachytene checkpoint does not require *RAD9*. The data suggest that at least part of the signal detected by the checkpoint in *dmc1* mutants is the accumulation of large amounts of ssDNA (29). Since recombination and the MI division need to be coordinated in normal WT cells, it has been suggested that the transient amount of ssDNA present in WT cells might set off the checkpoint system, causing a transient delay for the first division until recombination is completed.

The components of the initiation signal for delay of the first division. Because mutations completely deficient in the initiation of meiotic recombination go through the meiotic divisions, it had been thought that cells did not monitor initiation (41). Our previous work demonstrated that meiotic cells do monitor recombination initiation; they do so by assessing the presence of some of the meiotic recombination gene products required for initiation (15, 21). In the absence of *REC102*, *REC104*, *REC114*, and *RAD50* the first meiotic division actually begins at an earlier time. The presence of the proteins encoded by these genes results in a signal leading to the delay of the MI division in WT cells. The *SPO11* gene was also shown to be required for this normal MI delay (24). The work presented here confirms that the Spo11p is required for the MI delay and shows that the *MER2/REC107* gene product is required as well. The *MRE2* gene is needed for meiosis-specific splicing of *MER2/REC107* and *MER3* (a late recombination gene) (34, 35); it does not play a direct role in recombination initiation. A very low level of DSBs was detectable in an *mre2* mutant at the *his4*::*LEU2* locus (see Fig. 5 in reference 34). The *mre2* mutant undergoes MI at a time indistinguishable from the *rec104* mutant control. We presume that the early MI division in the *mre2* mutant is due to the reduction in the amount of the *MER2/REC107* gene product. The data also indicate that *rec103/ski8* mutants initiate the first division at the same time as a WT cell; like *MEI4*, *REC103/SKI8* is not needed for the signal to delay. Since the MI division starts at the same time as WT cells in both *rec103/ski8* and *mei4* mutants, recombination is not required for the normal delay.

HOP1 and *RED1* are also required for the MI division delay signal. These two components of the axial elements and the tripartite SC are required for full levels of recombination, although null mutants still display a large induction of recombination over the mitotic background level, reaching about 10% of the meiotic level mutants (19, 32, 40). Different levels of DSBs have been detected in *red1* and *hop1* mutants, although all experiments agree that the amounts of DSBs in *red1* and *hop1* mutants are reduced (32, 44, 52). Estimates of the

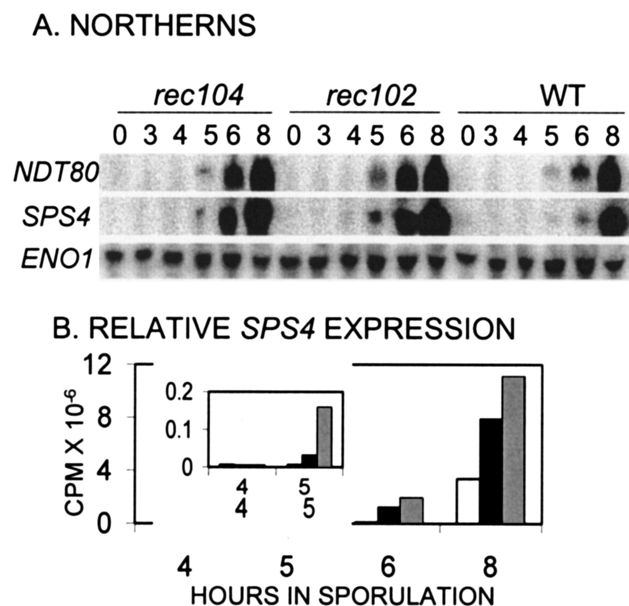


FIG. 8. Expression and activity of *NDT80* in Rec⁻ mutants undergoing an early first division. (A) The genotype of the strain examined is shown at the top. Numbers below the genotypes indicate the time in sporulation that RNA was isolated. The probes used for the Northern blots are shown to the left. (B) The amount of *SPS4* expression (corrected for loading by *ENO1*) is shown versus time. The white bar represents expression in WT cells, the black bar represents expression in *rec104* cells, and the gray bar represents expression in *rec102* cells.

level of DSBs in these mutants range from 5 to ~50% of normal levels: this range may reflect both strain differences and/or locus differences. It may also reflect the difference of monitoring DSBs by using a *rad50S* mutation versus a *com1/sae2* mutation. The MI division in *red1* and *hop1* mutants occurs at an early time indistinguishable from that of *rec104*. The normal timing of the MI division in *rec103/ski8* and *mei4* mutants indicates that DSBs are not necessary for the recombination initiation signal. The levels of DSBs in *hop1* and *red1* strains, along with the early division observed in those strains, strongly suggest that moderately high levels of meiotic DSBs are not sufficient either. At this time, the signal for recombination initiation that results in the normal delay of the first division consists of Rec102p, Rec104p, Rec107p, Rec114p, Spo11p, Rad50p, Hop1p, and Red1p.

Mechanisms for the initiation signal that delays the MI division. We address three possible mechanisms for the earlier start of the MI division in initiation mutants. The first hypothesis is that WT cells have a longer S phase because of the presence of recombination initiation proteins and that this results in a normal delay of the MI division. Cha et al. (4) showed that premeiotic S phase is shorter in *spo11* mutants than in WT cells (4). This is consistent with the view that the shorter S phase results in the earlier timing of the MI division observed in *spo11* mutants. The same authors, however, also showed that *rec102* mutants had normal timing of premeiotic S phase (4). This is something of a paradox, since *rec102* mutants clearly begin the MI division earlier than WT cells. We observe no significant differences in the timing of S phase (measured in three different ways) between WT cells and *rec102*, *rec104*, *rec114*, and *spo11* mutants (Table 1). We note that the exact time at which 50% of cells have entered S phase is slightly different depending, which of the two methods is used, but the conclusion that the mutants are the same as the WT remains the same. If the reason that the MI division starts earlier in recombination initiation mutants is that the premeiotic S phase is shorter, then it would seem that all Rec⁻ mutants displaying an earlier MI division should have a shorter S phase. We do not see it in our examination of four different mutants; Cha et al. (4) saw it in *spo11* mutants but not in *rec102* mutants. At the moment, we can only attribute the difference in the *spo11* phenotypes to strain differences. However, since *rec102* has normal S-phase timing in both strain backgrounds, even this explanation seems less than appealing. We conclude that neither the time of entry into S phase, the exit from 2C DNA content, nor the length of S phase is easily correlated with the time the MI division starts. We argue that alterations in S-phase length do not appear to explain the earlier MI division in recombination initiation mutants.

A second mechanism for the normal delay of MI caused by the presence of the recombination initiation functions was proposed by Shonn et al.(46). They suggested that the absence of chiasmata in *spo11* mutants (and presumably in all initiation mutants) resulted in immediate separation of chromosomes, since there would be no tension on the MI spindle. In WT Rec⁺ cells, the presence of chiasmata and the consequent tension would result in a delay of chromosome separation and hence the normal delay of the MI division. While appealing, we discuss four observations indicating that this hypothesis doesn't seem to explain our observations. First, this view suggests that spindles should form at the same time in Rec⁻ initiation mutants and in WT cells, but the chromosomes would be separated earlier in the mutants due to lack of chiasmata. Our data indicate that this is not the case. The MI spindle forms very early in *rec102* mutants, early in *rec104* mutants, and at the normal time in WT cells. This indicates that the delaying signal caused by recombination initiation functions acts prior to MI spindle formation. Second, both *rec103* and *mei4* mutants lack recombination but start the MI division at the same time as WT cells. Third, we have shown that *NDT80* expression and activity are delayed in WT cells compared to the initiation mutants, which display earlier divisions. Since *NDT80* is required for spindle formation for the first division, it is difficult to understand how the spindle checkpoint could monitor events where the spindle hasn't formed yet. Fourth, Shonn, et al. (46) showed that a *spo11 mad2* strain started the MI division at the same time as a *spo11* strain. Although the timing of a WT strain was not shown in the same experiment, we presume that the *spo11* mutant began MI earlier than a WT strain would have. We conclude that loss of the spindle checkpoint did not affect the early start of the MI division.

We note that Rec⁻ initiation mutants display another alteration of the MI division in addition to the earlier onset; the fraction of cells that are binucleate is higher and the number of binucleate cells persists longer. This is true even in a *rec103/ski8* mutant, which starts MI at the normal time. Shonn et al. (46) showed that a *spo11 mad2* strain had a lower level of binucleate cells than a *spo11* strain. From the data presented, it is harder to determine if the *mad2* mutation also reduced the persistence of binucleates. We propose that the spindle check-

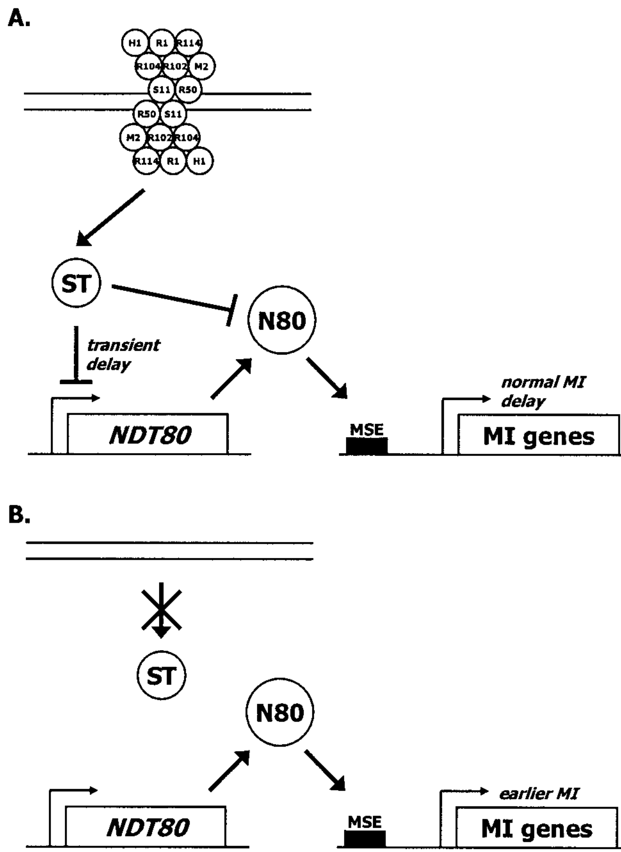


FIG. 9. Model for the earlier reductional division observed in some meiotic recombination initiation mutants. (A) The recombination initiation proteins necessary for the normal transient delay of the start of the MI division are shown on double-stranded DNA as a complex. The proteins are symbolized as follows: Spo11p, S11; Rad50p, R50; Rec104p, R104; Rec102p, R102; Mer2/Rec107p, M2; Rec114p, R114; Hop1p, H1; and Red1p, R1. The organization of the proteins is not intended to reflect the actual complex. Other recombination proteins (e.g., the protein coded for by *MEI4*) may well be present in the putative initiation complex, but are not shown either because mutations in them do not affect the start of the division or because they have not yet been tested. The recombination initiation complex on the DNA is relayed through an as yet unknown signal transduction pathway (ST). This pathway inhibits transcription of *NDT80* and (from the *SPS4* expression patterns) Ndt80p (N80) activity. Since the activity of Ndt80p is delayed, middle meiotic genes (MMGs) containing middle sporulation elements (MSEs) that require Ndt80p for expression are also delayed. Among the MMGs that are affected are genes required for the MI division. The delay in Ndt80p activity is transient, because the complex is transient and at least part of it leaves the DNA as recombination proceeds. (B) In the absence of the appropriate recombination initiation proteins, the complete complex does not form on DNA. Since no signal is detected, there is no inhibition of NDT80 expression or activity. Thus, the MMGs are expressed earlier, and the MI division begins at an earlier time.

point does not affect the start of the MI division, but does affect the ability of Rec$^-$ cells to proceed through the division.

A third mechanism for the normal delay of the MI division would be the presence of a putative recombination initiation complex that is recognized as a signal that recombination has started and that the MI division should be delayed (Fig. 9). This complex would consist of (at least) Rec102p, Rec104p, Rec107p, Rec114p, Spo11p, Rad50p, Hop1p, and Red1p. (Rec103p and Mei4p might be part of the complex, but would not be recognized by the signal transduction system.) If any of the eight proteins are missing, no delay occurs. Seven of these proteins are specifically produced in meiosis; Rad50p is also expressed in mitotic cells. Considerable support exists for interactions among these eight proteins. Spo11p and Rec102p have been shown to interact by high-copy suppression of specific alleles, negative interactions of specific alleles, and coimmunoprecipitation (22, 24). Rec102p and Rec104p have been shown to interact by allele-specific suppression, high-copy suppression of specific alleles, and coimmunoprecipitation (22, 43). Hop1p and Red1p have been shown to interact by high-copy suppression, allele-specific suppression, coimmunoprecipitation, and colocalization on the SC (9, 14, 20, 47). In addition, some *hop1* mutations can be suppressed by overexpression of *REC104* (14). Certainly the hypothesis that a recombination initiation complex exists (25) and is recognized as a signal seems both plausible and testable. It is not completely clear why *rad50* and *rec102* mutants undergo the first division even earlier than the other Rec$^-$ mutants: perhaps the putative initiation complex is built in stages, and defects in different steps have greater or lesser effects upon timing (21). If this view of the differences in early timing has merit, it suggests that the Rad50p and Rec102p might be early steps in assembly. The model also predicts that there should be functions that transduce the signal to the target(s).

Transduction of the initiation signal. Because components of the mitotic DNA damage checkpoint clearly play a role in arresting cells defective in some late recombination events, it seemed important to investigate whether they played a role in the MI division delay caused by the normal initiation signal. Because *RAD9* was not involved in the pachytene checkpoint, we hypothesized that it might instead have a role in the normal delay of MI caused by the presence of the early recombination functions. The predicted phenotype of a mutation in a gene involved in transducing the signal is an earlier first division. The data obtained show that *RAD9* is not required to transduce the signal; *rad9* mutants begin the first division indistinguishably from WT cells. Likewise, deletion of *RAD9* has no effect on the early division observed in a *rec104* mutant.

We next examined the effect of a *rad24* mutation on the timing of the first division. As with the prediction for *rad9*, *rad24* mutants should have had an earlier MI division if the Rad24p was required to transduce the initiation signal. This was not the case; *rad24* mutants actually go through the first division at a later time than WT cells. A similar result was recently reported by Shinohara et al. (45). From the timing of the *rec104 rad24* double mutant, it's clear that *rec104* is epistatic to *rad24*. This suggests that the delay in the first division observed in the *rad24* single mutant is solely due to presence of recombination. Shinohara et al. (45) suggested that the *rad24* observation calls into question the concept that the checkpoint acting in *dmc1* cells is also active in *DMC1* cells (as a delay). We had raised a similar concern (21). Since *rad24* mutants do not exhibit an earlier first division, it would appear that the simplest conclusion is that the pachytene checkpoint is not active in WT cells. This view is also consistent with the normal start of the MI division observed in *mei4* and *rec103/ski8* mutants; since recombination doesn't initiate, the putative *DMC1*

delay signal is never reached. In order to completely rule out the DNA damage checkpoint pathway as involved in signal transduction, we must examine the double *rad9 rad24* mutant. In the mitotic DNA damage checkpoint, both Rad9p and Rad24p have parallel and additive input into the subsequent steps of the pathway (8). Deletion of either one partially removes the ability to respond to DNA damage by arrest. The double mutant, however, is even more deficient in the checkpoint. It remains possible that both *RAD9* and *RAD24* must be removed in meiosis to eliminate transduction of the initiation signal and the consequent delay.

Target of the initiation signal. The discovery that the recombination initiation signal for MI division delay acts before the formation of spindles suggested that mutations in any target for the signal would block meiosis before division and before formation of MI spindles. The *NDT80* gene is just such a candidate. Null mutations in *NDT80* arrest in pachytene and although the spindle pole body duplicates, it does not separate and MI spindles do not form (53). The data in this paper indicate that Ndt80p is involved in the communication between recombination initiation functions and the MI division. The transcription of *NDT80* is increased at earlier times in both *rec102* and *rec104* mutants. *SPS4* is a middle meiotic gene regulated only by *NDT80* and not by other meiotic transcriptional regulators, and (36, 37; J. Segall, personal communication) *SPS4* transcription therefore measures active Ndt80p. *SPS4* is clearly transcribed at earlier times in *rec102* than in *rec104*, and earlier in *rec104* than in WT cells. The control of *NDT80* is complex and occurs at the level of transcription and posttranscriptional events (36). We are currently investigating known regulators of *NDT80* to determine which, if any, of them are involved in conveying the recombination initiation signal. The *SUM1* repressor of *NDT80* (28, 36) would seem one likely candidate.

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