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Meiotic Double-strand Break Repair Requires Two Nucleases, MRN and Ctp1, To Produce a Single Size-class of Rec12 (Spo11)-oligonucleotides

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Programmed DNA double-strand breaks (DSBs) in meiosis are formed by Spo11 (Rec12 in fission yeast), a topoisomerase II-like protein, which becomes covalently attached to DNA 5' ends. For DSB repair through homologous recombination, the protein must be removed from these DNA ends. We show here that Rec12 is endonucleolytically removed from DSB ends attached to a short oligonucleotide (Rec12-oligo complex), as is Spo11 in budding yeast. Fission yeast, however, has only one size-class of Rec12-oligo complexes, whereas budding yeast has two size-classes, suggesting different endonucleolytic regulatory mechanisms. Rec12-oligo generation strictly requires Ctp1 (Sae2 nuclease homolog), the Rad32 (Mre11) nuclease domain, and Rad50 of the MRN complex. Surprisingly, Nbs1 is not strictly required, indicating separable roles for the MRN subunits. Based on these and other data, we propose that Rad32 nuclease has the catalytic site for Rec12-oligo generation and is activated by Ctp1, which plays an additional role in meiotic recombination.

The repair of DNA double-strand breaks (DSBs) is essential for living cells. Faithful repair requires processing of the DSB to create a singe-stranded DNA end that can invade an intact homologous DNA template for repair. In some cases, a protein is bound to the DSB end and must be removed for repair to proceed. One notable example occurs during meiosis, when programmed DSBs are made by Spo11 (or its homolog), which becomes covalently linked to the 5' DSB ends (17). Removal of the protein is essential for repair of the DSBs and subsequent formation of crossovers, which are important for the proper segregation of homologs at the first meiotic division

as well as for the generation of genetic diversity. Removal of topoisomerases from DNA ends is also required for faithful repair when the topoisomerase reaction is aborted midway, as when cells are treated with topoisomerase inhibitors. Here, we address the mechanism of protein removal.

Meiotic recombination in the fission yeast *Schizosaccharomyces pombe*, is initiated by the formation of programmed DSBs by Rec12, its Spo11 homolog (4). To date, DSBs have been demonstrated by direct analysis of DNA only in *S. pombe* and the budding yeast *Saccharomyces cerevisiae*. This mechanism of recombination initiation (by DSB introduction) is thought to occur in most eukaryotes, since Spo11 homologs are widely conserved evolutionarily (17). After their formation, DSBs are repaired via homologous recombination. At least in *S. cerevisiae* the DNA ends are subjected to 5'→3' exonucleolytic resection to create 3' OH single-stranded DNA (ssDNA) overhangs (29). The resected DNA ends then are coated with a single-stranded DNA-binding protein and invade homologous duplex DNA by the action of Rad51, a RecA homolog, and its accessory proteins.

Spo11 and its homologs, including Rec12, have sequence similarity to a type II topoisomerase from archea, TopoVI (17). Consistent with this similarity, Spo11 and Rec12 introduce breaks in the DNA by a mechanism similar to that of topoisomerases: a dimer of the protein breaks both strands of the DNA molecule, and the conserved tyrosine of each monomer is covalently bound to the 5' end of each DNA strand. The *S. pombe* Rec12 protein, including its highly conserved tyrosine residue thought to be at the active site, is essential for meiotic recombination and DSB formation (7).

After the introduction of DSBs, Rec12, like Spo11, remains covalently attached to the DNA end (15). In order to process these meiotic DSBs into a substrate capable of

strand invasion, Rec12 has to be removed from the DNA end. A candidate for this removal is the MRN (Rad32-Rad50-Nbs1) nuclease complex, which is required for meiotic recombination. The requirement for the MRN complex in Spo11-mediated DSB formation in *S. cerevisiae* (3) complicates determining its role in DSB processing. The MRN complex is not, however, required for DSB formation in *S. pombe* (34), an outcome that enables us to determine the role of this complex in Rec12 removal from meiotic DSBs. A recent study implicated two putative nucleases, Ctp1 and Rad32 (Mre11 homolog), along with its partner protein Rad50, in the removal of Rec12 from the DNA end (14), but the mechanism of Rec12 removal was not determined, nor were other proteins tested for possible roles. In budding yeast Spo11 is removed by an endonucleolytic process, producing oligonucleotides of two distinct size-classes attached to Spo11 (23). We asked whether the same mechanism applies in S*. pombe* Rec12 removal. Here, we use an assay for the Rec12-oligonucleotide complex to determine the mechanism of this crucial step of meiotic recombination and discuss a model for the initial steps of this process in *S. pombe*.

MATERIALS AND METHODS

Induction and analysis of meiosis. The strains used are described in Table 1. Strains were grown, induced for meiosis, and analyzed for cellular DNA content and meiotic DNA breakage as described by Young *et al.* (35). Meiotic recombination and viable spore yields were conducted as described by Young *et al.* (34) and Ellermeier *et al.* (9).

Isolation of Rec12-oligo complexes. For protein extraction, cells containing Rec12-FLAG were opened by vigorous shaking with glass beads in ice-cold 10%

trichloroacetic acid (TCA; 1 ml/gm of wet cell paste) as described by Neale *et al.* (23). The precipitated proteins were solubilized in SDS extraction buffer (1 ml/gm of wet cell paste; ref. 23). Soluble protein (0.5 – 2.0 mg) was diluted 2-fold with 2× IP buffer (23) and incubated overnight at 4°C with 5 µg of monoclonal anti-FLAG antibody (clone M2; Sigma-Aldrich) bound to 25 µl of magnetic protein-G-agarose beads (Dynabeads®, Invitrogen) prewashed with BSA (5 mg/ml) in PBS. After collecting and washing with 1× IP buffer, the immune complexes were washed with TdT buffer (New England Biolabs) and incubated for 1 hr at 37°C in 12.5 µl 1x TdT buffer containing 0.5 mM $CoCl_2$, 5 units of terminal deoxynucleotidyl transferase (TdT; New England Biolabs), and 8 µCi of [α-^{32}P] dCTP (3000 Ci/mmol). Immune complexes were washed twice with 1× IP buffer and eluted by boiling in 2× Laemmli buffer (23). Eluted proteins were fractionated by SDS-PAGE using 10% pre-cast gels in Bis-Tris buffer (Invitrogen). Protein-oligonucleotide complexes were transferred to Immobilon-P membrane (Millipore). To detect the protein-bound oligonucleotide, the membrane was exposed to X-ray film and to a Typhoon storage phosphor imaging system (GE Healthcare). For western blot analysis, the membrane was probed with monoclonal anti-FLAG antibody conjugated to horseradish peroxidase (clone M2; Sigma, 1:3000 dilution) and detected using an ECL detection kit (Supersignal®, West Pico-Pierce).

Determination of oligonucleotide size. To determine the length of the Rec12-associated oligonucleotides, radio-labeled complexes prepared as above from 4.5 hr after meiotic induction were treated with Proteinase K (0.1 mg/ml; Roche) for 20 hr at 50ºC. An equal volume of 2× sequencing buffer (80% formamide, 10 mM EDTA, 0.1% xylene cyanol) was added to the Proteinase K reaction, which was boiled 2 min and chilled on ice. Samples were fractionated on a "sequencing" gel [20% polyacrylamide

(19:1 acrylamide:bisacrylamide), 7 M urea, 1× TBE, 18 cm long] at 700 volts for 1.5 h. Gels were exposed to Biomax supersensitive film (Kodak) for 72 hr as recommended by the manufacturer.

RESULTS

Rec12 is endonucleolytically removed from DSB ends. To determine whether Rec12 is removed from DSBs in *S. pombe* using a mechanism similar to that in *S. cerevisiae*, we induced meiosis in a synchronized culture of cells expressing the functional Rec12-FLAG protein (5). At various times during meiosis, cells were broken open in the presence of 10% trichloroacetic acid, to prevent degradation of intracellular intermediates and to precipitate proteins (23). After dissolving the proteins in SDS, Rec12-FLAG was precipitated with anti-FLAG antibodies. To determine whether Rec12 had DNA attached to it, the immunoprecipitates were incubated with [α-^{32}P] dCTP and terminal deoxynucleotidyl transferase (TdT), which labels 3' OH DNA ends. The components of the reaction mixture were resolved by SDS-PAGE and transferred to a membrane, which was analyzed by western blot analysis and autoradiography.

As expected, total Rec12-FLAG protein appeared 2 hr after meiotic induction, and its level increased during meiosis; the protein's mobility indicated a mass of ~47 kDa (Fig. 1). Three hr after induction of meiosis a [^{32}P] radioactive species appeared and persisted until at least 5 or 6 hr (Fig. 1A). The major Rec12-FLAG species migrated faster than the [^{32}P] radioactive signal, which presumably represents Rec12 bound to a short oligonucleotide. A fainter band detected by the antibody migrated with the radioactive signal, which we designate "Rec12-oligo" (Fig. 1B). Only about 10% of Rec12 protein was bound to the DNA. This result suggests that Rec12 is involved in steps of meiosis other than DSB formation, such as chromosome segregation (8, 27).

To determine if the appearance of the Rec12-oligo is dependent on DSB formation, we tested a mutant lacking Rec10, a linear element protein essential for DSB formation (10). Although Rec12 protein was readily detected by anti-FLAG antibody, no radioactive species was detectable (Fig. 2A). Thus, as expected, Rec12-oligo formation ("clipping") depends on DSB formation.

Rec12-oligo is kinetically competent to be an intermediate of meiotic recombination. To be a kinetically competent intermediate of meiotic recombination, the Rec12-oligo should appear after the formation of DSBs and before the first meiotic division. To test this expectation, we studied the kinetics of Rec12-oligo formation in relation to pre-meiotic DNA replication, DSB formation, and the two meiotic divisions (Fig. 1D). DNA replication, monitored by flow cytometry (data not shown), occurred between 2 and 3 hr after meiotic induction. DSBs at the meiotic hotspot *mbs1*, detected by Southern blot hybridization, were first visible 3 hr after meiotic induction, were maximal at 3.5 hr, and largely disappeared by 5 hr (Fig. 1C). As assayed by microscopy of stained nuclei, meiosis I and II were nearly completed by ~5 hr and ~6 hr, respectively (Fig. 1D). These events occurred at approximately the times reported previously (6). The maximal abundance of Rec12-oligo was about one hour after maximal DSB abundance and about one-half hour before meiosis I (Fig. 1C, D, and E). The appearance of this species between DSB formation and meiosis I makes it kinetically competent to be a recombination intermediate. From this outcome and the genetic requirements for its production (see also below), we conclude that Rec12-oligo formation is an essential step in meiotic recombination.

A single size-distribution of Rec12-oligo suggests a non-conserved regulation of endonucleolytic cleavage in meiosis. In *S. cerevisiae* meiosis, Spo11

attached to oligonucleotides appears as two distinct nucleoprotein complexes with different electrophoretic mobilities (23). After release from Spo11 by proteolysis, the oligonucleotides migrate during gel electrophoresis also as two distinct size-classes, approximately 12 – 26 nt and 28 – 34 nt long. In contrast, a similar analysis of *S. pombe* Rec12 revealed only one size-class of Rec12-oligo complexes (Fig. 1A).

To test further that there is indeed only one size-class of complexes and to determine the length of the attached oligonucleotides, we treated the [^{32}P] labeled Rec12-oligo complexes with Proteinase K to degrade the covalently attached Rec12 protein and resolved the [^{32}P] labeled oligonucleotides on a denaturing ("sequencing") gel. After Proteinase K treatment, we detected only one size-class of oligonucleotides (Fig. 3). The mode of the distribution was ~22 nt, and the range was ~13 – 29 nt. These results show that indeed only one size-class of Rec12-oligo complex is formed in *S. pombe*. We discuss the significance of this result later.

The nuclease activity of the MRN complex is required for Rec12 clipping. To determine the enzymes required for formation of Rec12-oligo, we tested a series of mutants deficient in nucleases that were previously implicated in the initial steps of homologous recombination (7). The MRN complex, composed of Rad32, Rad50, and Nbs1, is a strong candidate for this reaction. In the *rad50S* missense mutant with an altered MRN complex, Rec12 forms DSBs but remains covalently bound to the DNA (15). As expected, we could not detect the Rec12-oligo in this mutant at any time after induction, although Rec12 protein was induced to the level seen in wild type (Fig. 4A and B). We could, however, detect the radioactive signal in a wild-type strain analyzed in parallel (Fig. 4A).

Next, we tested whether the putative nuclease active site of the MRN complex, which resides in Rad32 (33), is required for Rec12-oligo generation. The *rad32-D65N* mutant is equivalent to the *S. cerevisiae mre11-D56N* mutant, which lacks nuclease activity but forms an intact MRN (MRX) complex (19, 20); the *S. pombe rad32-D65N* mutant also forms the MRN complex (25). In this putative nuclease-deficient mutant Rec12-oligo was not detectable (Fig. 5A), although Rec12 was expressed and DSBs were formed and persisted to at least 6 hr after induction (Fig. 5B and unpublished data). Rad32 nuclease activity *per se* is not sufficient for Rec12-oligo generation, since *rad50Δ* and *nbs1Δ* strains were also deficient in Rec12-oligo formation, although Rec12 was expressed and DSBs were formed in these mutants (Fig. 6A-D). In summary, the MRN subunits Rad50 and Nbs1 and the putative nuclease active site in Rad32 are required for clipping off Rec12. Below, we show that Nbs1 is not, however, strictly required for clipping.

The putative nuclease Ctp1 is also essential for Rec12-oligo generation. Ctp1, also called Nip1 (1, 22), is a conserved protein important for DSB repair through homologous recombination in *S. pombe.* Its homolog Sae2 in budding yeast has nuclease activity *in vitro* (21) and is required for Spo11-oligo formation in *S. cerevisiae* (23). It is not known, however, whether the nucleolytic activity of Sae2 is required for Spo11 clipping. CtIP, the human homolog of Ctp1, is essential for DSB resection in mitotic cells (26). To determine if Ctp1 is also essential for the formation of Rec12-oligo, we tested a *ctp1Δ* mutant and found that Rec12-oligo was not generated, although, as for the *MRN* mutants, Rec12 was expressed and DSBs were formed and persisted beyond their normal time of repair (Fig. 7). These results show that two

putative nucleolytic proteins – Ctp1 and Rad32 – are required for Rec12 clipping. We discuss the implications of this unexpected outcome later.

We tested another nuclease, exonuclease 1 (Exo1), which specifically digests double-stranded DNA in the 5'→3' direction (30), as would be required to convert meiotic DSBs into invasive DNA ends with long 3' ss tails. We found that an *exo1Δ* mutant was fully proficient in forming Rec12-oligo (Fig. 8). We discuss below a possible role for Exo1 in meiotic recombination.

Nbs1 and Rad50 are differentially required for viable spore formation and meiotic recombination. Since MRN and Ctp1 are required for Rec12 clipping, we would expect each of the four polypeptides to be required for meiotic recombination and generation of viable spores. As expected, *rad50Δ, rad32Δ,* and *ctp1Δ* mutants produced <0.2% of the viable spore yield of wild type (10, 22, 34) (Table 2). Unexpectedly, the *nbs1Δ* mutant produced 26% of the wild-type viable spore yield. These crosses were done at 25°C, the standard temperature for *S. pombe* meiotic crosses (13). Since we assayed DNA intermediates, including Rec12-oligo, in synchronized meiotic cultures at 34°C, we repeated these crosses at 34°C. At this temperature *nbs1Δ* yielded markedly fewer viable spores (1.2% of wild type) but still >8-fold more than *rad50Δ, rad32Δ,* or *ctp1Δ* at 25°C. Thus, the Rad50 and Rad32 subunits of the MRN complex and Ctp1 are more stringently required for producing viable spores than is Nbs1, although all four subunits appear indispensable for producing Rec12-oligo, at least at 34°C (Figs. 5A, 6A, 6C, and 7B; see also next section). Our assay for Rec12-oligo may not be sufficiently sensitive, however, to detect the low level of Rec12-oligo expected in the *nbs1Δ* mutant at 34°C (a few percent of the wild-type level, based on the viable spore yield).

To determine the requirements for the MRN complex and Ctp1 in meiotic recombination, we measured both crossing over (as *ade6 – arg1* intergenic recombination) and gene conversion (as *ade6* intragenic recombination). While at 25°C crossing over was reduced by more than a factor of 70 in *rad50Δ, rad32Δ,* and *ctp1Δ* mutants, it was reduced by only a factor of ~6 in *nbs1Δ* at both low and high temperatures. Gene conversion was also reduced by a factor of ~6 in *nbs1Δ* at 25°C (Table 2). The low viable spore yields in the *rad50Δ, rad32Δ,* and *ctp1Δ* mutants did not allow a reliable estimate of gene conversion at either temperature; the same was true for *nbs1Δ* at high temperature. In summary, Rad50, Rad32, and Ctp1 are strongly required for formation of viable spores and meiotic recombinants, but, surprisingly, Nbs1 is less strictly required, especially at low temperature.

Nbs1 is not strictly required for Rec12-oligo generation. To test the suggestion that Nbs1 is not strictly required for Rec12 clipping at low temperature, we induced a culture for meiosis at 34°C and then reduced the temperature to 25°C at 3.5 or 4 hr, when most DSBs have formed and Rec12-oligo generation has begun (Fig. 9). With this regimen, we detected a low level of Rec12-oligo in the *nbs1Δ* mutant at 25°C, but none in the *rad50Δ* mutant (Fig. 9). The level of Rec12-oligo at 5.5 hr was similar to the level of viable spore yield, about ¼ of that of wild type (Table 2). These results show that, as predicted, Nbs1 is not strictly required for Rec12 clipping, although Rad50 appears to be. We propose below an explanation for the differential requirements for the MRN subunits in meiotic recombination and DNA break repair.

DISCUSSION

We report here that fission yeast Rec12 is clipped off the end of meiotic DSBs by the action of an endonuclease, producing a short oligonucleotide covalently linked to Rec12. This endonucleolytic cleavage requires the nuclease activity of the Rad32 (Mre11) component of the MRN complex and the Ctp1 putative nuclease (Table 3). The regulation of this key step in meiotic recombination appears to be distinctly different from that reported in budding yeast (23). We discuss below the implications of these and other results for the processing of meiotic DSBs and the formation of recombinants.

Rec12 is removed from DSB ends via endonucleolytic cleavage. Although the biochemical mechanism of meiotic DSB formation in *S. pombe* seems to be similar or identical to that in *S. cerevisiae*, many aspects of the regulation of DSB formation and repair are not conserved between these two distantly related yeasts (7). *S. cerevisiae* Spo11 is endonucleolytically clipped from the DNA end with a spectrum of short oligonucleotides attached to it; this spectrum is bimodal in length, with modes of ~15 and ~30 nucleotides (23). We found that *S. pombe* Rec12 is also removed from DNA attached to a spectrum of oligonucleotides, suggesting that the basic endonucleolytic mechanism of removal is conserved. However, we found a unimodal distribution of oligonucleotide lengths, with a mode of ~22 nucleotides (Fig. 3). While the molecular basis of this difference is not currently clear, it may be that in *S. cerevisiae* some protein excludes endonucleolytic cleavage of DNA ~20 – 25 nucleotides from Spo11; this protein may not be present at meiotic DSBs in *S. pombe*. This suggestion is consistent with certain proteins, notably the MRN complex, being required for DSB formation in *S. cerevisiae* but not in *S. pombe* (7).

An additional dramatic difference in the regulation of DSB end processing in these yeasts is the time between DSB formation and removal of Spo11 or Rec12. *S.*

pombe Rec12-oligo was most abundant about an hour after maximal DSB abundance (Fig. 1D and E), indicating a distinct lag between DSB formation and clipping off Rec12. In *S. cerevisiae,* Spo11-oligo formation occurs nearly immediately after DSB formation (23). This difference in timing accounts for the ability to detect Rec12 covalently bound to DNA in wild-type (*i.e., rad50*$^{+}$) *S. pombe* (15) but apparently not in *S. cerevisiae* (18). These differences parallel the requirement for the MRN complex in DSB formation in *S. cerevisiae* but not in *S. pombe* (3, 34). Thus, in *S. cerevisiae* the MRN complex is in place to remove Spo11 immediately after the DSB is formed, whereas in *S. pombe* DSBs are formed without MRN, which may take an hour to be recruited to DSBs or activated to remove Rec12.

Two putative nucleases are required for Rec12 clipping. Two proteins with a putative nuclease activity, Ctp1 and Rad32, are required for the formation of Rec12-oligo (Fig. 5A and 7B). Hartsuiker *et al.* (14) recently reported that both proteins are required for the disappearance of Rec12-DNA complexes detected by antibody to epitope-tagged Rec12, but the product of this reaction was not determined. In their study, as in ours (Fig. 5A), the *rad32-D65N* mutant, which lacks the putative nuclease active site (33) but forms the MRN complex (25), was as defective as a *rad32Δ* mutant. Ctp1 was also required in both studies. Ctp1 is a distant homolog of *S. cerevisiae* Sae2, which has nuclease activity *in vitro* (21), and of human CtIP, which in combination with a low level of Mre11-Rad50 (MR) has nuclease activity lacking with either single protein preparation (26). Thus, we infer that two nucleases are required for a simple endonucleolytic cleavage.

To account for this conundrum, we propose that Rad32, as part of the MRN complex, is the endonuclease that clips off Rec12 and that Ctp1 is required for the

activity of Rad32. This proposal is based in part on our recent report that meiotic recombination stimulated by a protein-free DSB is not blocked by the *rad32-D65N* mutation but is blocked by *ctp1Δ* (11). In this case, DSB resection but not protein removal is required for recombination. We propose that Ctp1 is the major resecting nuclease in *S. pombe* meiosis and that its binding near a DSB end enables Rad32 to clip off Rec12. We further propose that the MRN complex activates Ctp1, because Exo1, rather than Ctp1, is the major resecting nuclease in the absence of Rad50 (11). In *S. pombe* Rec12 makes DSBs in the absence of MRN or Ctp1 (1, 34) (Figs. 5B, 6B, 6D and 7C). We propose that after DSB formation MRN and Ctp1 bind and positively regulate each other. This mechanism would coordinate Rec12 clipping and DNA resection for rapid and efficient DSB repair and recombination.

The role of Nbs1 differs from that of Rad50 and Rad32. As expected from the failure of Rec12 to be removed from DSB ends in *rad50Δ* and *rad32-D65N* mutants (Fig. 5A and 6A), *rad50Δ* and *rad32Δ* mutants formed <0.2% as many viable spores as wild type (Table 2) (10, 34). Unexpectedly, however, *nbs1Δ* mutants formed at least eight times more viable spores than *rad50Δ* or *rad32Δ* mutants; at low temperature the yield in *nbs1Δ* was even greater – about ¼ that of wild type and >150 times higher than that of *rad50Δ* or *rad32Δ*. At low temperature the *nbs1Δ* mutant produced readily detectable Rec12-oligo, whereas the *rad50Δ* mutant did not; at high temperature, neither mutant produced detectable Rec12-oligo (Figs. 6A, 6C, and 9). To our knowledge, this is the first report of distinct phenotypes of null (deletion) mutations in the *MRN* genes in *S. pombe*. In *S. cerevisiae xrs2Δ* strongly reduces binding of Mre11 to DNA at a meiotic DSB hotspot, but *rad50Δ* only partially reduces this binding (2).

The curious temperature-sensitivity of *nbs1Δ* suggests that some function can at least partially substitute for Nbs1 at low temperature but not at high temperature. One possibility, which we favor, is that MR can at least partially function without Nbs1 at low temperature, but at high temperature MR requires Nbs1 to stabilize or activate MR. The MRN complexes of the two yeasts discussed here may differ in their requirement for an additional subunit to stabilize or activate the MR complex at low temperature. Consistent with our proposal, purified human Nbs1 stabilizes MR complexes (24). Additional experiments may support this suggestion and reveal other differences in the roles of the MRN subunits in DSB repair and recombination.

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Figure legends

FIG. 1. Rec12 Is endonucleolytically removed from DNA at the time expected for a meiotic recombination intermediate. (A and B) Equal amounts of protein from meiotic cell extracts prepared at the indicated time after meiotic induction of strain GP6232 (*rec12-201::6His-2FLAG*) were immunoprecipitated and labeled with TdT and [α-^{32}P] dCTP. The membrane was exposed to a Phosphorimager to detect ^{32}P (A) and then probed with an anti-FLAG antibody conjugated to horseradish peroxidase to detect Rec12-FLAG (B). Filled arrowhead, Rec12 covalently bound to a short oligonucleotide (Rec12-oligo). Open arrowhead, unbound Rec12 protein. The species with mobilities lower than those indicated by arrowheads may be Rec12 bound to DNA broken at nearly random sites (see also Fig. 2). (C) DNA was digested with NotI, subjected to pulsed-field gel electrophoresis, and Southern blot-hybridized with a probe from the left end of NotI fragment J, as described by Young *et al.* (35). Arrows indicate chromosome I NotI fragment J (top) and its two prominent DNA fragments broken at meiotic break sites *mbs1* and *mbs2*. (D) Kinetics of appearance of Rec12-oligo (red circles) during meiosis. Orange triangles, percentage of cells with G2 DNA content, as measured by flow cytometry (data not shown). Green squares, DSBs at meiotic break site *mbs1* on NotI fragment J of chromosome I. Blue diamonds, (non-normalized) percentage of cells

with two nuclei (MI); brown squares, percentage of cells with four nuclei (MII). (E) Reproducible kinetics of appearance of Rec12-oligo during meiosis. Data were normalized to the highest observed value for each experiment (at 4 or 5 hr) set at 100%. Data points are the mean ± SEM (n = 4).

FIG. 2. Rec12-mediated DSB formation is required for formation of Rec12-oligo. Meiotic induction of a *rec10Δ* mutant strain (GP6733; panel A) and a control strain (GP4117) with wild-type Rec12 (no FLAG tag; panel B). Samples were treated as described for Fig. 1. Filled arrowhead, [^{32}P] Rec12-oligo complex. Open arrowhead, Rec12 free protein. The faint, low-mobility [^{32}P]-labeled species in B may be Rec12-DNA complexes broken at nearly random sites and bound non-specifically to the antibody beads (see also Fig. 1). "+" indicates *rec10^{+} rec12-FLAG* cell extract (strain GP6010 at 4 hr).

FIG. 3. A single size-distribution of Rec12-oligo. Rec12-oligo complex was prepared 4.5 hr after meiotic induction as in Fig. 1. The protein-DNA complexes were treated (+) or not (-) with Proteinase K. The reaction mixture was analyzed on a "sequencing" gel alongside a set of [5'-^{32}P] oligonucleotide markers (Invitrogen; lane m) and autoradiographed. Filled arrowhead, Rec12 covalently bound to a short oligonucleotide (Rec12-oligo complex).

FIG. 4. The *rad50S* mutation blocks Rec12-oligo formation. Meiotic induction of a *rad50^{+}* control strain GP6010 (panel A) and *rad50S* mutant strain GP6013 (panel B). Samples were treated as described for Fig. 1. Filled arrowhead, [^{32}P] Rec12-oligo complex. Open arrowhead, Rec12 free protein.

FIG. 5. The nuclease activity of the MRN complex is necessary for Rec12-oligo formation. (A) Meiotic induction of nuclease-deficient mutant *rad32-D65N* mutant strain

GP6677. Samples were treated as described for Fig. 1. "+" indicates *rad32*$^{+}$ cell extract of strain GP6010 at 4 hr. Filled arrowhead, [^{32}P] Rec12-oligo complex. Open arrowhead, Rec12 free protein. (B) DSB analysis of DNA from the meiotic induction analyzed in panel A. Arrows, chromosome I NotI fragment J (top) and its two prominent DNA fragments broken at meiotic break sites *mbs1* and *mbs2*.

FIG. 6. Intact MRN complex is required for Rec12-oligo formation. Meiotic inductions of a *rad50Δ* mutant strain (GP6730; panels A and B) and an *nbs1Δ* mutant strain (GP6892; panels C and D). Samples were treated as described for Fig. 1. Filled arrowhead, [^{32}P] Rec12-oligo complex. Open arrowhead, Rec12 free protein. "+" indicates *rad50*$^{+}$ *nbs1*$^{+}$ extracts of strain GP6010 at 4 hr. (B and D) DSB analysis of DNA from the meiotic inductions analyzed in panels A and C, respectively. Arrows, chromosome I NotI fragment J (top) and its two prominent DNA fragments broken at meiotic break sites *mbs1* and *mbs2*.

FIG. 7. Ctp1 is a second putative nuclease necessary for Rec12-oligo formation. Meiotic induction of *ctp1*$^{+}$ control strain GP6010 (panel A) and *ctp1Δ* mutant strain GP6654 (panel B). Samples were treated as described for Fig. 1. Filled arrowhead, [^{32}P] Rec12-oligo complex. Open arrowhead, Rec12 free protein. (C) DSB analysis of DNA from the meiotic induction analyzed in panel B. Arrows, chromosome I *Not*I fragment J (top) and its two prominent DNA fragments broken at meiotic break sites *mbs1* and *mbs2*.

FIG. 8. Exonuclease I is dispensable for Rec12-oligo formation. Meiotic induction of *exo1Δ* mutant strain GP6653. Samples were treated as described for Fig. 1. Filled arrowhead, [^{32}P] Rec12-oligo complex. Open arrowhead, Rec12 free protein.

FIG. 9. Nbs1 is not strictly required for Rec12-oligo formation. Strains GP6010 (*nbs1*$^{+}$), GP6892 (*nbs1Δ*), and GP6730 (*rad50Δ*) were induced as in Fig. 1 at 34°C (labeled H) and cooled to 25°C (labeled C) at 3.5 hr. Samples were treated as described for Fig. 1. Filled arrowhead, [^{32}P] Rec12-oligo complex. Open arrowhead, Rec12 free protein. A repeat of this experiment gave similar results (data not shown).

FIG. 10. Model for the initial steps of meiotic DSB repair in *S. pombe.* (1) Rec12 and other proteins bind to the DNA after premeiotic replication and generate a DSB. Rec12 remains covalently bound to the DNA ends. (2) The Rad32 nuclease, as part of the MRN complex and activated by Ctp1, removes Rec12 bound to a short oligonucleotide. Failure to remove Rec12 from the DNA ends causes persistence of the breaks. (3) The DSB ends are resected by Ctp1, activated by MRN, to create 3' OH ssDNA tails. (4) The ssDNA tails are coated by Rad51 and invade intact DNA (not shown) to repair the DSB and produce homologous recombinants.

Table 1. *S. pombe* strains

Strain	Genotype[a]
GP13	*h⁻ ade6-52*
GP1293	*h⁺ ade6-M26 arg1-14*
GP4117	*h⁻ pat1-114 ade6-3049*
GP5374	*h⁻ ade6-52 rad50S*
GP6010	*h⁺ rec12-201::6His-2FLAG pat1-114 ade6-3049*
GP6013	*h⁺ rec12-201::6His-2FLAG pat1-114 rad50S ade6-3049*
GP6232	*h⁻/h⁻ ade6-3049/ade6-3049 pat1-114/pat1-114 rec12-201::6His-2FLAG/rec12-201::6His-2FLAG +/his4-239 lys4-95/+*
GP6653	*h⁻ rec12-201::6His-2FLAG pat1-114 ade6-3049 exo1-1::ura4⁺*
GP6654	*h⁺ rec12-201::6His-2FLAG pat1-114 ade6-3049 ctp1::kanMX6*
GP6677	*h⁺ rec12-201::6His-2FLAG pat1-114 ade6-3049 rad32-D65N*
GP6730	*h⁺ rec12-201::6His-2FLAG pat1-114 ade6-3049 rad50::kanMX6*
GP6733	*h⁺ rec12-201::6His-2FLAG pat1-114 ade6-3049 rec10-175::kanMX6*
GP6848	*h⁻ ade6-52 nbs1::kanMX6*
GP6849	*h⁻ ade6-52 rad50::kanMX6*
GP6853	*h⁺ ade6-M26 arg1-14 nbs1::kanMX6*
GP6892	*h⁺ rec12-201::6His-2FLAG pat1-114 ade6-3049 nbs1::kanMX6*
GP6927	*h⁺ ade6-M26 arg1-14 ura4-D18 rad32::ura4*
GP6928	*h⁻ ade6-52 ura4-D18 rad32::ura4*
GP6930	*h⁺ ade6-M26 arg1-14 ctp1::kanMX6*
GP6932	*h⁻ ade6-52 ctp1::kanMX6*
GP6934	*h⁺ ade6-M26 arg1-14 rad50S*
GP6950	*h⁺ ade6-M26 arg1-14 rad50::kanMX6*

[a] Alleles are described in the following references: *rec12-201::6His-2FLAG* (5), *pat1-114* (16), *ade6-3049* (28), *rad50S* (12), *rad50::kanMX6* (12), *exo1-1::ura4⁺* (31), *ctp1::kanMX6* (22), , *rec10-175::kanMX6* (10), *nbs1::kanMX6* (32), *rad32-D65N* (33).

Table 2. Differential requirements for Nbs1 *vs.* Rad50, Rad32, and Ctp1 in viable spore formation and meiotic recombination

Mutant[a]	Viable spore yield (% of wild type)[b,c]	*ade6 – arg1* intergenic recombination (cM)[c,d]	*ade6* intragenic recombination ($Ade^+/10^6$ viable spores)[c]
+	≡100	74 ± 13	3500 ± 490
+ (34°C)	≡100	54	4500
nbs1Δ	26 ± 5	10 ± 1.6	580 ± 40
nbs1Δ (34°C)	1.2 ± 0.3	10 ± 2.5	—[e]
rad50S	30 ± 3	64 ± 21	3700 ± 280
rad50Δ	0.15	0.7 ± 0.1	—[e]
rad32Δ	0.09	1.0 ± 1.5	—[e]
ctp1Δ	0.002 ± <0.0001	1	—[e]

[a] Crosses were conducted at 25°C, except as noted.

[b] In wild type, 2.0 ± 0.5 viable spores per viable cell were obtained at 25°C, and 0.15 at 34°C.

[c] Mean ± SEM from 4 – 33 crosses except wild type at 34°C.

[d] Spore colonies (470 – 2348) from 4 – 13 crosses were tested for *ade6 – arg1* recombinants, except for wild type at 34°C (1 cross, 140 colonies) and *ctp1Δ* (33 crosses, 204 colonies; the intergenic recombination data were pooled). Frequencies were converted to cM using Haldane's equation.

[e] The viable spore yield was too low for an accurate determination.

Table 3. Summary of gene products required for Rec12-oligo formation

Mutant	DSB formation	DSB repair	Rec12-oligo formation
+	+	+	+
rad50S	+[a]	–[a]	–
rec10Δ	–[b]	NA	–
rad32-D65N	+	–	–
rad50Δ	+[c]	–[c]	–
nbs1Δ	+	–[d]	Ts
ctp1Δ	+	–	–
exo1Δ	ND	ND	+

"+", DSBs or Rec12-oligo are formed; "–", not formed; "NA", not applicable; "ND", not determined; “Ts”, temperature-sensitive. [a] Ref. 35. [b] Ref. 10. [c] Ref. 34. [d] At 34°C.