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# The Third Exon of the Budding Yeast Meiotic Recombination Gene *HOP2* Is Required for Calcium-dependent and Recombinase Dmc1-specific Stimulation of Homologous Strand Assimilation\*

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**Background:** The previously characterized version of the *Saccharomyces cerevisiae* recombination protein Hop2 was a mutant.

**Results:** Wild type Hop2-Mnd1 robustly stimulates the activity of the meiotic strand exchange protein Dmc1 but not that of the mitotic strand exchange protein Rad51.

**Conclusion:** Hop2-Mnd1 specifically activates Dmc1 at physiological concentrations of ATP/Mg<sup>2+</sup>/Ca<sup>2+</sup>.

**Significance:** Robust Dmc1 activity allows further reconstitution of meiotic recombination.

During meiosis in *Saccharomyces cerevisiae*, the *HOP2* and *MND1* genes are essential for recombination. A previous biochemical study has shown that budding yeast Hop2-Mnd1 stimulates the activity of the meiosis-specific strand exchange protein ScDmc1 only 3-fold, whereas analogous studies using mammalian homologs show >30-fold stimulation. The *HOP2* gene was recently discovered to contain a second intron that lies near the 3'-end. We show that both *HOP2* introns are efficiently spliced during meiosis, forming a predominant transcript that codes for a protein with a C-terminal sequence different from that of the previously studied version of the protein. Using the newly identified *HOP2* open reading frame to direct synthesis of wild type Hop2 protein, we show that the Hop2-Mnd1 heterodimer stimulated Dmc1 D-loop activity up to 30-fold, similar to the activity of mammalian Hop2-Mnd1. ScHop2-Mnd1 stimulated ScDmc1 activity in the presence of physiological (micromolar) concentrations of Ca<sup>2+</sup> ions, as long as Mg<sup>2+</sup> was also present at physiological concentrations, leading us to hypothesize that ScDmc1 protomers bind both cations in the active Dmc1 filament. Co-factor requirements and order-of-addition experiments suggested that Hop2-Mnd1-mediated stimulation of Dmc1 involves a process that follows the formation of functional Dmc1-ssDNA filaments. In dramatic contrast to mammalian orthologs, the stimulatory activity of budding yeast Hop2-Mnd1 appeared to be specific to Dmc1; we observed no Hop2-Mnd1-mediated stimulation of the other budding yeast strand exchange protein Rad51. Together, these results support previous genetic experiments indicating that Hop2-Mnd1 specifically stimulates Dmc1 during meiotic recombination in budding yeast.

Meiosis is a sexual reproduction pathway in which diploid cells undergo two specialized rounds of chromosome segregation to form haploids. Prior to the first round of meiotic chromosome segregation, homologous chromosomes are replicated and then undergo high levels of homologous recombination. Recombination involves the transfer and exchange of information between DNA molecules. Meiotic recombination is initiated by induction of programmed double-stranded breaks (DSBs),<sup>2</sup> which are formed by the transesterase activity of the Spo11 protein (1, 2). The DSB ends of DNA molecules are resected by nucleolytic processing to generate 5' duplex junctions with 3' single-stranded DNA (ssDNA) tails. In the budding yeast *Saccharomyces cerevisiae*, Dmc1 and Rad51 bind the ssDNA tails to form nucleoprotein filaments; Dmc1 catalyzes the homology search and strand exchange processes with Rad51 acting as a regulatory co-factor (3–5). Dmc1 filaments search the genome for a homologous DNA sequence and catalyze strand exchange, resulting in heteroduplex product. The *in vitro* displacement loop (D-loop) assay models the *in vivo* strand exchange reaction using a supercoiled dsDNA plasmid and a homologous ssDNA molecule as substrates.

When assayed alone, the D-loop activity of Dmc1 is low compared with that of the bacterial recombinase RecA (6, 7). However, genetic and biochemical studies have provided evidence for key co-factors that stimulate the activity of Dmc1 (5, 8–12). Among these co-factors are the Hop2 and Mnd1 proteins, which form a heterodimer, Hop2-Mnd1. Hop2-Mnd1 has been shown to interact with Dmc1 to promote strand exchange (13–18). Furthermore, genetic studies have shown that both Hop2 and Mnd1 are required for meiotic homologous recombination; *dmc1*, *hop2*, and *mnd1* mutants display similar phenotypes; meiotic DSBs form, and the mutant cells arrest in prophase (13, 16, 19–21). Cytological studies show that both

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) KJ489455.

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<sup>2</sup> The abbreviations used are: DSB, double-stranded break; D-loop, displacement loop; Sc, *Saccharomyces cerevisiae*.

Rad51 and Dmc1 form DNA double-stranded break-dependent immunostaining foci in *hop2* or *mnd1* mutants, but the breaks are not repaired and no strand exchange intermediates are formed (13, 16, 19–21).

The original mapping of budding yeast *HOP2* transcripts was flawed because the method used did not take into account the possibility that the gene contained a second intron near its 3'-end (19). This led to the incorrect annotation of the C-terminal sequence of the coding region and to subsequent biochemical analysis of a mutant form of the protein (14). Recent studies provide evidence that *HOP2* has two introns<sup>3</sup> (see supplemental Tables 6 and 7 in Ref. 22). Here we show that both *HOP2* introns are efficiently spliced during meiosis, forming a transcript coding for a different C-terminal sequence than that characterized previously. We further show that the wild type ScHop2 protein, coded by three exons, as a component of the Hop2-Mnd1 heterodimer can dramatically enhance the D-loop activity of Dmc1. Additional characterization reveals similarities as well as a critical difference between the biochemical activity of budding yeast Hop2-Mnd1 and its mammalian counterparts.

## EXPERIMENTAL PROCEDURES

**DNA Substrates**—The supercoiled plasmid pRS306 was purified without alkali denaturation by fractionation via cesium chloride equilibrium density gradient centrifugation (6). DNA pRS306.90 is a 90-mer oligonucleotide synthesized by Integrated DNA Technologies. This oligo is homologous to the DNA sequence 764–854 in plasmid pRS306 (23) and was 5' <sup>32</sup>P-labeled by a standard protocol. The unreacted [ $\gamma$ -<sup>32</sup>P]ATP in the labeling reactions was removed by a Sephadex G25 microspin column (GE Healthcare), and residual T4 polynucleotide kinase was heat-inactivated. The PCR primers (from Integrated DNA Technologies) (Fig. 1, *B* and *C*) have DNA sequences from the *ScHop2* gene and are as follows: *a*, 5'-aagctaacgtgaactgaccag-3'; *b*, 5'-gtagcagcgtatcttcagacattatccgc-3'; *c*, 5'-cgctcttgactctgtaactaagaac-3'; and *d*, 5'-taagattat-tacctatataatttgataataatgc-3'.

**Reverse Transcription-Polymerase Chain Reaction**—RT-PCR was employed to determine whether the *ScHOP2* mRNA differs in size from its gene. First, total RNA (24) and genomic DNA were prepared from *S. cerevisiae* SK1 mitotic and meiotic cells with methods that have been described previously (25, 26). Meiotic cells were harvested after 6 h in sporulation medium. Second, complementary DNA (cDNA) was generated by M-MLV reverse transcriptase using total RNA as template and oligo(dT)<sub>20</sub> as primer by following a protocol provided by the enzyme vendor (Promega). Third, genomic DNA or total cDNA was used as template in PCR reactions with the primer pairs indicated in Fig. 1, *B* and *C*. The PCR-generated DNA products were analyzed by electrophoresis in 1.2% agarose gel with standard 1× TAE buffer (40 mM Tris acetate (pH 7.5) and 1 mM EDTA).

**Plasmid Construction, Expression, and Protein Purification**—The coding sequences (ORFs) of *S. cerevisiae* *HOP2* and *MND1* were sequentially inserted into the expression vector

pETDuet-1 (Novagen). First, the ORF of *HOP2* was amplified by RT-PCR from its mRNA (from yeast SK1 strain) and hexahistidine-tagged at its N terminus. The PCR-generated DNA fragment was inserted into the expression vector at restriction sites NcoI and HindIII to form the plasmid pETDuet-1-Hop2. Second, the ORF of *MND1* was amplified by PCR using plasmid pTFW515 (14) as template. The resulting DNA fragment was inserted at the NdeI and XhoI sites of pETDuet-1-Hop2 to form the final expression plasmid pNRB662 (or pETDuet-1-ScHop2-Mnd1), which harbors both the *HOP2* and the *MND1* coding sequences, and confirmed by sequence analysis.

*S. cerevisiae* Hop2 and Mnd1 proteins were coexpressed from pNRB662 in *Escherichia coli* cells Rosetta(DE3)pLysS using the T7 promoter system as described previously (5). In this system, only Hop2 is hexahistidine-tagged, and Mnd1 was co-purified through its interaction with Hop2. In brief, bacterial lysate with expressed Hop2-Mnd1 was prepared by French press and cleared by centrifugation. The Hop2-Mnd1 complex was then purified from the lysate in sequential steps by nickel resin (GE Healthcare) in batch followed by His-Trap FF in FPLC (GE Healthcare). The eluted protein fractions were pooled, concentrated by Centricon (Millipore), and exchanged into storage buffer (25 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM EDTA, 100 mM NaCl, and 10% glycerol (v/v)) by Sephadex G25 column. All steps were carried out at 4 °C.

**Other Proteins**—*S. cerevisiae* proteins Dmc1, Rad51, and Rad52 were purified as described previously (5). Rad54 was a gift from Wolf Heyer, at the University of California, Davis, CA.

**D-loop Assay**—The reaction (final volume 10  $\mu$ l) was in a buffer containing 25 mM Tris-HCl (pH 7.8), 1 mM DTT, 5 mM MgCl<sub>2</sub>, 3 mM ATP, 0.25 mM CaCl<sub>2</sub>, and 60 mM NaCl. 0.25 mM Ca<sup>2+</sup> is the concentration in standard yeast growth media. ScDmc1 or ScRad51 (1.5  $\mu$ M) was first incubated with <sup>32</sup>P-labeled pRS306.90 ssDNA (40 nM 90-mer, or 3.6  $\mu$ M nucleotides) at 37 °C for 5 min followed by the addition of 1  $\mu$ l of supercoiled plasmid pRS306 (5 nM or 22  $\mu$ M for base pairs) with or without 1  $\mu$ l of ScHop2-Mnd1 (at the concentration indicated in the Fig. 2–7 legends). The mixture was further incubated to initiate D-loop formation at 37 °C for 10 min. When included, ScHop2-Mnd1 was preincubated with plasmid (in buffer without Ca<sup>2+</sup> unless specified) at room temperature for 2 min prior to mixing with nucleoprotein filaments. The D-loop reaction was stopped by the addition of 2  $\mu$ l of SDS (1% w/v) and proteinase K (1 mg/ml) followed by incubation at 37 °C for 5 min for deproteinization. The samples were analyzed by electrophoresis in 1% agarose gel in 1× TAE buffer. The gel was dried onto a positively charged DEAE membrane (Roche Diagnostics), exposed to an image plate, and analyzed using the Molecular Dynamics Storm 860 PhosphorImager (Amersham Biosciences) and the computer software Quantity One. Quantification of radioactive image intensities was performed with data within a linear range of exposure. The D-loop yield was expressed as a percentage of input plasmid DNA.

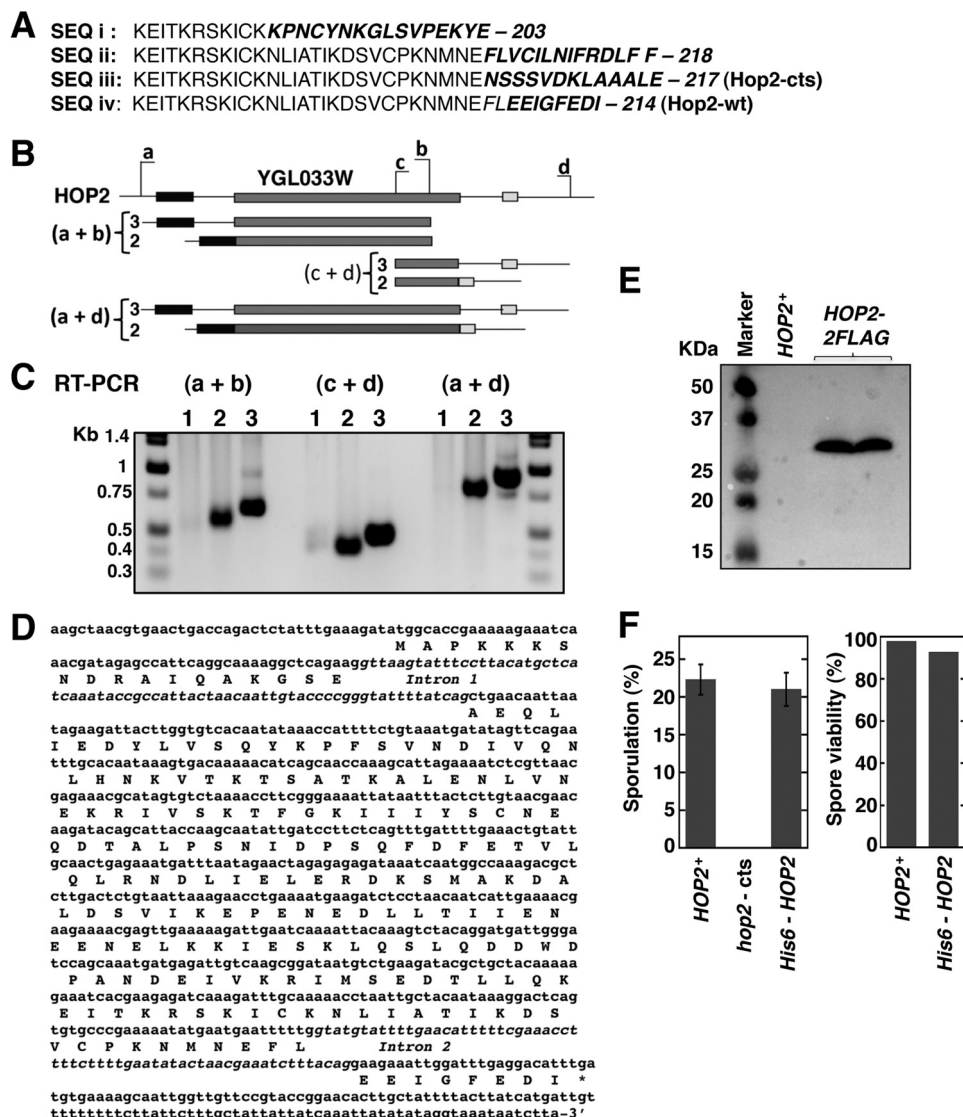
The D-loop reactions involving ScRad54/ScRad51 did not contain calcium. D-loop formation was at 37 °C for 7 min.

**Yeast Strains, Culture, and Western Blot**—All strains are derivatives of SK1. Standard techniques for yeast cultures

<sup>3</sup> D. Qin and J. Staley, unpublished data.



## Dmc1-specific Stimulation by Hop2-Mnd1



**FIGURE 1. The two introns in the ScHOP2 gene are removed during pre-mRNA splicing to reveal a novel C-terminal sequence.** *A*, the C-terminal sequence of different versions of ScHop2: SEQ i (Leu *et al.* (19)); SEQ ii (Stanford Saccharomyces Genome Database); SEQ iii (pTFW515, this work); SEQ iv (this work). The number of amino acids in each ScHop2 sequence is indicated at the end of its respective C terminus. *B*, the physical map of the ScHOP2 gene and diagrams illustrating PCR products. Exons are depicted by black or gray boxes; introns and non-coding gene regions are depicted by solid lines. The letters *a*, *b*, *c*, and *d* stand for PCR primers, and their relative positions are marked. The numbers 2 and 3 represent PCR reactions described in *C*. *C*, agarose gel image of RT-PCR reaction products analyzed by electrophoresis. The inverted image of an ethidium bromide-stained gel is shown. Lane 1, the PCR template was total cDNA from mitotic culture; lane 2, template was cDNA from 6-h meiotic culture; lane 3, template was genomic DNA. The primer pairs for PCR were as indicated. *D*, the nucleotide sequence of the ScHOP2 gene and the deduced amino acid sequence. The DNA sequences of the two introns are in *italics*. *E*, Western blot analysis showing ScHop2-2FLAG protein is expressed in meiotic cells. Two consecutive FLAG tags were cloned in-frame with the novel C terminus encoded by the third exon of ScHOP2. Yeast meiotic diploid cell lysates from ScHOP2<sup>+</sup> and ScHOP2-2FLAG from two independent cultures were analyzed. The detection was with anti-FLAG tag antibody (Sigma-Aldrich). *F*, his6-ScHop2 functions *in vivo*. Sporulation efficiency (error bars represent S.E., *n* = 200) and spore viability were determined for yeast diploid strain expressing His<sub>6</sub>-tagged ScHop2.

and sporulation were used (4). The His6-ScHOP2 and ScHOP2-2FLAG strains were constructed by overlap-PCR using an hphNT1 module followed by LiAc transformation (4). Properly targeted integrations were confirmed by PCR analysis and DNA sequencing. Details of strain construction are available upon request. Western blot detection was by anti-FLAG antibody following the standard protocol of the vendor (Sigma-Aldrich).

## RESULTS

*Efficient Splicing of the Two Introns of the HOP2 Transcript—*  
 The original characterization of the budding yeast HOP2 gene

was complicated by three errors. First, the method used to map the 3'-end of HOP2 transcripts assumed that no second intron was present and, as a consequence, incorrectly assigned the Hop2 C-terminal sequence (Fig. 1*A*, SEQ i). Second, a frame-shift sequencing error was present in the originally submitted sequence that became apparent when a number of additional sequences (Fig. 1*A*, SEQ ii) were submitted to the database. A third error occurred during the construction of a bacterial expression plasmid (pTFW515) designed to express SEQ ii, which lacks the third exon. Recent sequence analysis showed that pTFW515 carries a substitution mutation at the C-terminal sequence (Fig. 1*A*, SEQ iii). This mutation was not detected

previously because the plasmid was generated as part of a large-scale subcloning project in which the sizes of inserted restriction fragments and induced proteins were used as quality controls rather than DNA sequencing (27). During efforts to correct the defect in pTFW515, it became clear that the protein corresponding to the open reading frame predicted from *SEQ ii* is highly toxic in *E. coli*.<sup>4</sup> Thus, it is likely that the mutation carried by pTFW515 was selected, following transformation of a ligation mixture, because it directed expression of a less toxic form of the protein. pTFW515 was used for production of the Hop2-Mnd1 protein that was characterized in a previous study (14).

We carried out a genome-wide search for previously undetected introns using a scheme that identified intron lariats. This approach provided evidence for a second intron at the 3'-end of *HOP2*, making it one of only eight budding yeast genes with more than one intron. A published study that profiled full-length cDNAs also found evidence for a second intron in *HOP2*, although this information was not annotated in the *Saccharomyces* Genome Database (see supplemental Tables 6 and 7 in Ref. 22).

To better characterize *HOP2* transcripts from meiotic cells, and to determine whether both *HOP2* introns are efficiently spliced, we carried out RT-PCR analysis on meiotic RNA using primer pairs designed to anneal with predicted exons and that flanked both the 5' and 3' introns (Fig. 1B). We first confirmed that intron 1 was removed from the *HOP2* mRNA (Fig. 1C) as reported by Leu *et al.* (19, 28). The DNA fragment generated by PCR with the primer pair (*a + b*) using total cDNA from meiotic cells as template was shorter than that generated using genomic DNA as template (Fig. 1C, compare lanes 2 and 3). The sequences of these DNA fragments were determined. The shorter fragment has 563 bp (Fig. 1C, lane 2), which is 70 bp shorter than the 633 bp (lane 3) generated using genomic DNA as template. Therefore, the first intron of *HOP2* is 70 bp and is absent from meiotic mRNA. Using the same RT-PCR strategy with primer pair *c + d* (Fig. 1C), the shorter fragment has 408 bp (lane 2), which is 62 bp shorter than the 470 bp (lane 3) generated using genomic DNA as template. Therefore, *HOP2* has a second intron of 62 bp. When primer pair *a + d* was used, the product was 798 bp (Fig. 1C, lane 2), 132 bp shorter than the product generated using genomic DNA as template. Therefore, the major RNA template in these reactions is one in which both introns in the *HOP2* pre-mRNA are spliced. The splicing of both introns was very efficient as evident from the single major PCR product produced when meiotic cDNAs were the templates (Fig. 1C, lanes 2). Little or no PCR product was generated when total cDNA from mitotic cells was used as template (Fig. 1C, all lanes 1), confirming that *HOP2* expression is meiosis-specific (14, 19).

After removal of both introns, the mature *HOP2* mRNA codes for a protein of 214 residues with a predicted molecular mass of 24,478 daltons (Fig. 1D, GenBank<sup>TM</sup> accession number KJ489455). The newly discovered third exon of *HOP2* encodes eight amino acids (Fig. 1, A, *SEQ iv*, and D). To verify whether

Hop2 with this new C terminus was made as predicted, two FLAG tags were fused in tandem to exon 3 of *ScHOP2* to code for a protein that would be 16 amino acids larger (26,469 daltons) and would be detected by anti-FLAG antibody if expressed in-frame with the new C terminus. Yeast meiotic whole cell lysates from diploids of *ScHOP2*<sup>+</sup> and *ScHOP2*-2FLAG were prepared for Western blot detection. As shown in Fig. 1E, only the lysates expressing *ScHOP2*-2FLAG reacted with the anti-FLAG tag antibody, providing direct evidence that ScHop2 with the new C terminus was expressed *in vivo* during meiosis.

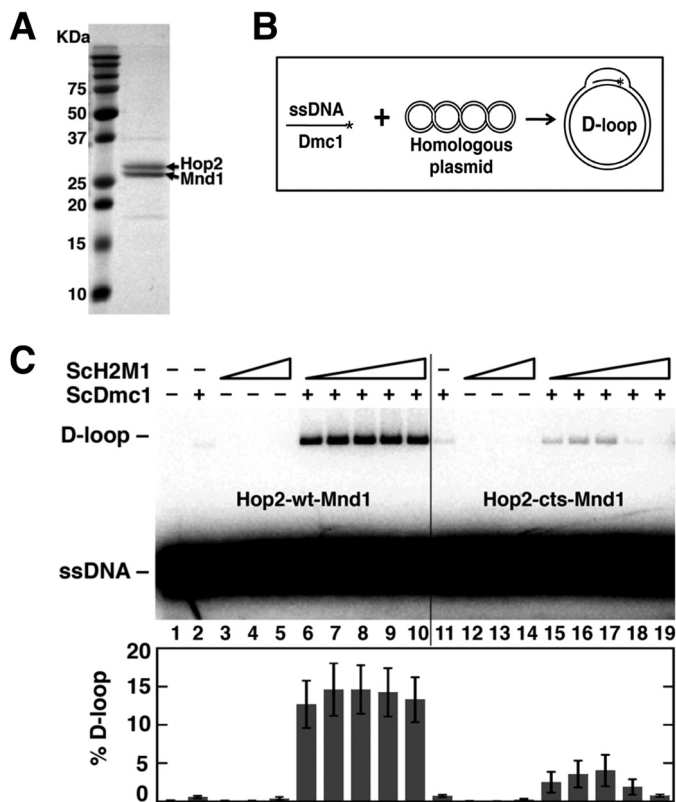
We will refer to the protein encoded by the complete gene as ScHop2-wt (or simply ScHop2) and the defective protein used in the previous study as ScHop2-cts (C-terminal substitution mutation).

*The ScHop2 C-terminal Sequence Is Important for Robust ScHop2-wt-Mnd1-mediated Stimulation of ScDmc1-mediated D-loop Formation*—To test whether the normal C-terminal sequence of Hop2 makes a significant contribution to the activity of ScHop2-Mnd1, we compared the activity of ScHop2-wt-Mnd1 to that ScHop2-cts-Mnd1 protein. An intronless copy of *HOP2* was cloned into an expression vector that carries a copy of *MND1*, to allow for co-purification of the two proteins. Purification was facilitated by an N-terminal hexahistidine tag (His<sub>6</sub> tag) added to the *HOP2* coding sequence. A diploid yeast strain homozygous for His<sub>6</sub>-Hop2 showed normal sporulation efficiency and spore viability (Fig. 1F). Therefore, the His<sub>6</sub>-Hop2 fusion protein is functional *in vivo* with no apparent defect. Following co-expression, we found that ScHop2 protein co-purified with ScMnd1 protein as a stable protein heterodimer (Fig. 2A), as observed previously for both ScHop2-cts-Mnd1 and the mammalian Hop2-Mnd1 heterodimer (14, 29, 30).

Purified ScHop2-wt-Mnd1 and ScHop2-cts-Mnd1 were tested for the ability to stimulate ScDmc1-mediated strand exchange in standard D-loop assays (Fig. 2B). ssDNA was added at an 8-fold molar excess to target dsDNA. This condition was determined to be optimal under our assay condition for detecting Dmc1 activity in the absence of stimulatory factors. ScHop2-cts-Mnd1 stimulated ScDmc1 activity by only 2–4-fold, yielding about 1–4% D-loop (Fig. 2C, compare lanes 15–17 with lane 11). This low level of stimulation is similar to that reported previously (14). In dramatic contrast, we found that ScHop2-wt-Mnd1 stimulated Dmc1 D-loop activity up to 30-fold with final D-loop yields of 10–20% of input dsDNA substrate (Figs. 2C, lanes 6–10, and 6A, lanes 4–7). The stimulation of ScDmc1 is saturated at the range of 0.25 to 1 μM ScHop2-Mnd1. The high level of stimulation of ScDmc1 by ScHop2-wt-Mnd1 is comparable with the level of stimulation observed with the mammalian counterparts (15). Importantly, a diploid strain homozygous for the *hop2-cts* was unable to support spore formation *in vivo*, despite its modest ability to stimulate Dmc1 *in vitro* (Fig. 1F). These findings demonstrate that the Hop2 C-terminal sequence is critical to the function of Hop2-Mnd1. We also found that binding of Hop2 to Mnd1 in the ScHop2-cts-Mnd1 heterodimer was less stable than in the ScHop2-wt-Mnd1 heterodimer; Mnd1 dissociated from column-bound ScHop2-cts, but not column-bound ScHop2-wt, during the extensive high salt wash used for purification (data

<sup>4</sup> Y.-L. Chan and N. Handa, unpublished data.

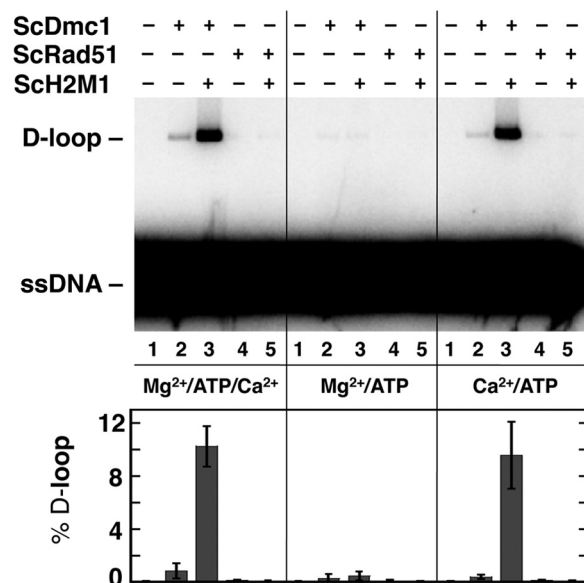
## Dmc1-specific Stimulation by Hop2-Mnd1



**FIGURE 2. Comparison of ScDmc1 strand exchange stimulated by ScHop2-Mnd1 bearing different ScHop2 C-terminal sequences.** *A*, Coomassie Blue-stained SDS-polyacrylamide gel showing the purified yeast ScHop2-Mnd1 complex (3  $\mu$ g of proteins). *B*, scheme of the D-loop assay. *C*, gel image from D-loop assay with results graphed. ScDmc1 concentration was 1.5  $\mu$ M in the D-loop reactions. The concentrations of ScHop2-Mnd1 (ScH2M1) were 0.125, 0.5, and 2  $\mu$ M for lanes 3–5 and 12–14; and 0.125, 0.25, 0.5, 1, and 2  $\mu$ M for lanes 6–10 and 15–19. Error bars represent S.D.,  $n = 4$ .

not shown). This result suggests that the C-terminal sequence of Hop2 is important for binding Mnd1. Hereafter we refer to ScHop2-wt-Mnd1 simply as ScHop2-Mnd1.

**ScHop2-Mnd1 Promotes Mainly the Homology Search and Strand Invasion Steps of ScDmc1-mediated Strand Exchange Activity**—Calcium ion ( $\text{Ca}^{2+}$ ) has been shown to enhance the homology-dependent activities of mammalian Rad51 as well as mammalian and budding yeast Dmc1 (31–35). Stimulation by  $\text{Ca}^{2+}$  was shown to be associated with inhibition of the DNA-dependent ATPase of the protein and with the formation of longer more stable nucleoprotein filaments through  $\text{Ca}^{2+}$ -induced conformational changes (33, 34). Hop2-Mnd1 has been shown to enhance hDmc1 and hRad51 activity by two distinct functions, first by stabilization of Dmc1-ssDNA filaments, in a manner that could be analogous to the effect of  $\text{Ca}^{2+}$ , and second by non-homology-dependent capture of duplex DNA following assembly of ssDNA nucleoprotein filaments (17, 36). To determine the relationship between the mechanisms through which  $\text{Ca}^{2+}$  and Hop2-Mnd1 stimulate Dmc1, we measured the level of stimulation of Dmc1 by Hop2-Mnd1 in the presence of different metal co-factors. We assessed the ScHop2-Mnd1 stimulation of ScDmc1 D-loop forming activity in the presence of  $\text{Mg}^{2+}$  alone,  $\text{Mg}^{2+}$  plus  $\text{Ca}^{2+}$ , or  $\text{Ca}^{2+}$  alone (Fig. 3). We found that ScHop2-Mnd1 stimulated ScDmc1 D-loop formation only when  $\text{Ca}^{2+}$  was included in the filament formation



**FIGURE 3. ScHop2-Mnd1 stimulation of ScDmc1-mediated strand exchange is facilitated by  $\text{Ca}^{2+}$ .** The effect of ScHop2-Mnd1 (0.5  $\mu$ M) on D-loop formation mediated by either ScDmc1 (1.5  $\mu$ M) or ScRad51 (1.5  $\mu$ M) in buffers containing 5 mM  $\text{Mg}^{2+}$ , 5 mM  $\text{Mg}^{2+}$  with 0.25 mM  $\text{Ca}^{2+}$ , or 1 mM  $\text{Ca}^{2+}$  were analyzed. The image shown was from the D-loop assay with results from three replicates graphed. Error bars represent S.D.,  $n = 3$ .

buffer (Fig. 3, lanes 1–3). A 23-fold stimulation was seen comparing  $\text{Mg}^{2+}$  with  $\text{Mg}^{2+}$  plus 0.25 mM  $\text{Ca}^{2+}$ ; a higher concentration of  $\text{Ca}^{2+}$  (1 mM) did not further enhance the levels of D-loop formation. However, in the absence of  $\text{Ca}^{2+}$ , no appreciable amount of D-loop was formed with or without ScHop2-Mnd1 (Fig. 3, the  $\text{Mg}^{2+}$ /ATP panel, lanes 2 and 3). These results suggest that Hop2-Mnd1-mediated stimulation of Dmc1 requires filament stabilization by  $\text{Ca}^{2+}$ .

**ScHop2-Mnd1 Stimulates ScDmc1 D-loop Formation at Physiological Concentrations of ATP,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$** —As demonstrated above, ScHop2-Mnd1 cannot stimulate ScDmc1-mediated D-loop formation unless  $\text{Ca}^{2+}$  is present. Although intracellular concentrations of  $\text{Mg}^{2+}$  range from 4 to 30 mM, often in complex with ATP (1–10 mM) (37, 38), intracellular free  $\text{Ca}^{2+}$  concentrations range from a baseline of around 0.1  $\mu$ M up to 10  $\mu$ M during calcium influx (39). Therefore, the 250  $\mu$ M  $\text{Ca}^{2+}$  (equivalent to  $\sim 160$   $\mu$ M free  $[\text{Ca}^{2+}]_i$ ) present in the D-loop assays described above, as well as the mM  $\text{Ca}^{2+}$  concentrations typically used by others, are much higher than the physiological level. (The concentration of free  $[\text{Ca}^{2+}]_i$  excludes the  $\text{Ca}^{2+}$ -ATP form and was calculated using the program WEBMAXC STANDARD with the following parameters: temperature 37°C, pH 7.8, ionic strength 50 mM, ATP concentration 3 mM, and  $\text{Mg}^{2+}$  concentration 5 mM.) Nonetheless,  $\text{Ca}^{2+}$  has been reported to play a role in the early stages of meiosis (40, 41) and in response to DNA damage in mammalian cells (42). We therefore investigated whether ScHop2-Mnd1 could stimulate ScDmc1 D-loop activity at or near physiological  $\text{Ca}^{2+}$  concentrations of 1 to 17  $\mu$ M (the equivalent of 0.6–10  $\mu$ M free  $[\text{Ca}^{2+}]_i$ ) (39).

We measured ScHop2-Mnd1 stimulation of ScDmc1 D-loop formation at various concentrations of total  $\text{Ca}^{2+}$  with  $\text{Mg}^{2+}$  and ATP fixed at 5 and 3 mM, respectively (Fig. 4). We found



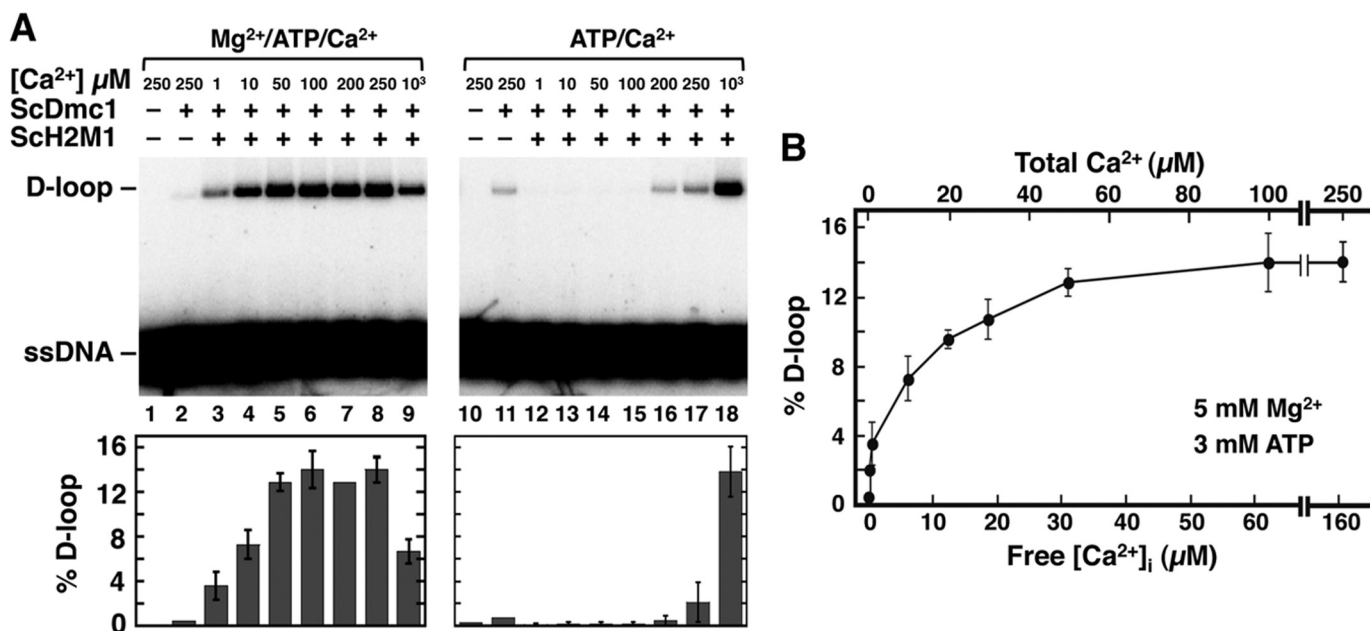


FIGURE 4. ScHop2-Mnd1 stimulates ScDmc1-mediated D-loop formation at physiological concentrations of Ca<sup>2+</sup>. *A*, gel images from D-loop assays; both panels were from the same gel. The results from three or more replicates were graphed. Error bars represent S.D. D-loops were formed by ScDmc1 (1.5 μM) in the presence of ScHop2-Mnd1 (0.5 μM) with increasing concentrations of Ca<sup>2+</sup> (as indicated) in buffers containing 3 mM ATP with or without 5 mM Mg<sup>2+</sup>. *B*, the results from Mg<sup>2+</sup>/ATP/Ca<sup>2+</sup> D-loop reactions, similar to those shown in *A* but involving a wider range of Ca<sup>2+</sup> concentrations (0, 0.1, 1, 10, 20, 30, 50, 100, and 250 μM). Error bars represent S.D., *n* = 3.

that stimulation was initiated at a Ca<sup>2+</sup> concentration of 0.1 μM, reached maximum levels between 50 and 100 μM, and declined at 1 mM (Fig. 4, *A*, lanes 3–9, and *B*). About 7% of the D-loops were formed at 10 μM Ca<sup>2+</sup> (equivalent to 6.1 μM free [Ca<sup>2+</sup>]<sub>i</sub>), which is within the physiological level, and this activity was about 50% of the maximum stimulation of ~14% D-loop. However, when Mg<sup>2+</sup> was omitted from parallel reactions (Fig. 4*A*, lanes 12–18), stimulation reached maximum levels only at 1 mM Ca<sup>2+</sup> and was not detected at Ca<sup>2+</sup> concentrations below 200 μM. This observation suggests that ScHop2-Mnd1-mediated stimulation of ScDmc1 activity *in vitro* requires a much higher Ca<sup>2+</sup> concentration if Mg<sup>2+</sup> is not present. Notably, less D-loop activity was observed with 1 mM Ca<sup>2+</sup> in the presence of 5 mM Mg<sup>2+</sup> as compared with 250 μM Ca<sup>2+</sup> and 5 mM Mg<sup>2+</sup> (Fig. 4*A*, compare lane 9 with lane 8). This result suggests that at very high concentrations, Ca<sup>2+</sup> may compete with Mg<sup>2+</sup> for ATP and/or replace Mg<sup>2+</sup> in binding to ScDmc1. Clearly, stimulation of ScDmc1-mediated D-loop formation by ScHop2-Mnd1 is effective in the presence of physiological levels of all three components: ATP, Mg<sup>2+</sup>, and Ca<sup>2+</sup>.

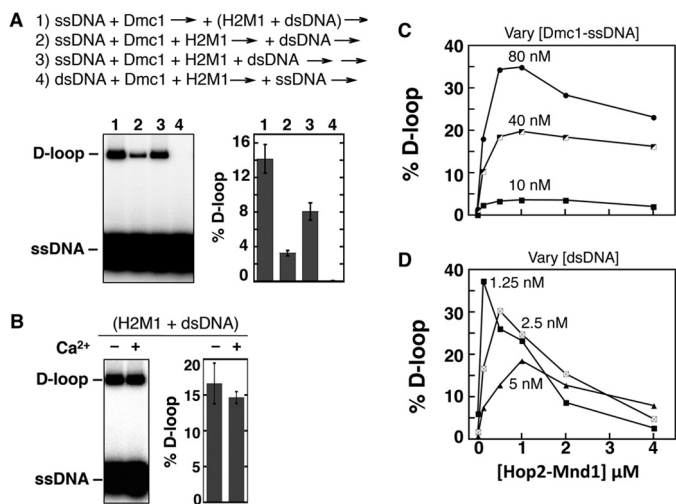
*ScHop2-Mnd1 Interacts with dsDNA to Exert Its Stimulatory Effect on ScDmc1-mediated Strand Exchange*—*In vitro*, Hop2-Mnd1 binds to both ssDNA and dsDNA with a preference for the latter (14, 30). The reaction requirements described in Fig. 3 suggest that ScHop2-Mnd1 stimulation is likely to occur after ScDmc1 has formed a filament on ssDNA. If this interpretation is correct, then the order of addition of DNA substrates could affect the stimulatory activity. Indeed, we found that ScHop2-Mnd1 stimulated ScDmc1 D-loop formation most effectively when it was preincubated with supercoiled plasmid dsDNA prior to addition of preformed ScDmc1-ssDNA filament (Fig. 5*A*, reaction 1); about 12–16% of the D-loops were formed when this order of addition was followed. This preference is

explained if ScHop2-Mnd1 facilitates the capture of a ScDmc1-ssDNA filament after it has bound dsDNA. This mechanism was proposed previously for the ScHop2-cts-Mnd1 protein (14) and the mammalian proteins (17).

If all reaction components were added before incubation to allow D-loop formation (Fig. 5*A*, reaction 3), the yield of D-loops was reduced to 7–9%, about 50% lower than the yield seen when Hop2-Mnd1 was preincubated with dsDNA separately. If ScHop2-Mnd1 and ScDmc1 were preincubated with ssDNA prior to dsDNA addition (Fig. 5*A*, reaction 2), the yield of D-loops was 3%, 4–5-fold lower than optimal levels. In this reaction, ScHop2-Mnd1 is likely to have competed with ScDmc1 for ssDNA. Finally, when ScHop2-Mnd1 and ScDmc1 were preincubated with dsDNA prior to ssDNA addition (Fig. 5*A*, reaction 4), no D-loops were detected, consistent with previous studies showing that allowing Dmc1 (or Rad51) to bind dsDNA prior to the addition of ssDNA blocks D-loop formation (6, 43, 44). The D-loop activity was not affected by the presence or absence of Ca<sup>2+</sup> in the dsDNA-Hop2-Mnd1 preassembled complex as long as the Dmc1-ssDNA filament was assembled in buffer containing Ca<sup>2+</sup> (Fig. 5*B*).

Further mechanistic analysis was carried out to determine whether the optimal amount of ScHop2-Mnd1 would titrate with the amount of ScDmc1-ssDNA filament or dsDNA (Fig. 5, *C* and *D*). We found that the optimal ScHop2-Mnd1 concentration titrated only with dsDNA but not with ScDmc1-ssDNA filaments. Although the mechanism through which Hop2-Mnd1 inhibits D-loops at high concentration remains to be determined, we speculated that excess binding of Hop2-Mnd1 to the dsDNA target duplex may either directly mask the target sequence or force the duplex plasmid into a conformation that is not amenable to strand assimilation.

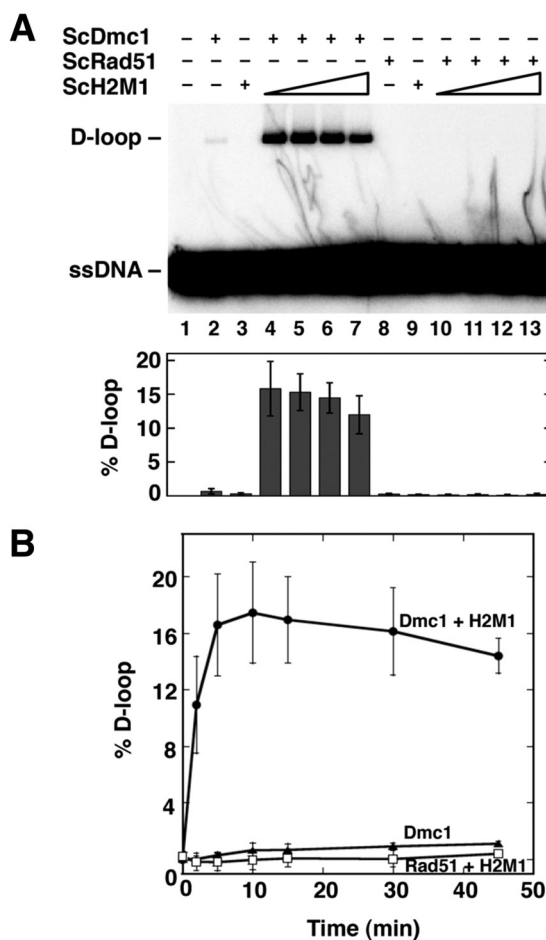
## Dmc1-specific Stimulation by Hop2-Mnd1



**FIGURE 5. The order of DNA addition affects ScDmc1-mediated D-loop formation in the presence of ScHop2-Mnd1.** *A*, the order of addition of ssDNA (3.6  $\mu\text{M}$  nt) or supercoiled plasmid DNA (dsDNA, 22  $\mu\text{M}$  bp) was as indicated in each D-loop reaction, which had both  $\text{Mg}^{2+}$  (5 mM) and  $\text{Ca}^{2+}$  (0.25 mM) in the buffer. D-loops were formed by ScDmc1 (1.5  $\mu\text{M}$ ) in the presence of ScHop2-Mnd1 (0.5  $\mu\text{M}$ ). Reaction components were mixed in the order indicated. The *first arrow* in each reaction indicates the incubation of mixed reactants at 37 °C for 10 min followed by the *second arrow*, which indicates an additional incubation at 37 °C for 15 min. ScHop2-Mnd1 and dsDNA in *reaction 1* were preincubated without  $\text{Ca}^{2+}$  at room temperature for 3 min prior to adding it to Dmc1-ssDNA filaments. *Error bars* represent S.D.,  $n = 3$ . *B*, calcium has no significant effect on Hop2-Mnd1 interaction with dsDNA during D-loop formation. Prior to initiation of D-loop formation, ScHop2-Mnd1 was preincubated with dsDNA in the presence or absence of  $\text{Ca}^{2+}$  (0.25 mM) before adding it to ScDmc1-ssDNA filaments preassembled in buffer containing  $\text{Ca}^{2+}$ . *Error bars* represent S.D.,  $n = 3$ . *C*, optimal amount of ScHop2-Mnd1 does not titrate with Dmc1-ssDNA filaments. Three different amounts of ScDmc1-ssDNA filaments were assayed. The filaments were preassembled with ScDmc1 on ssDNA (10, 40, and 80 nM) using the ratio of 3 nt to 1 ScDmc1. D-loop activity was determined at a fixed dsDNA plasmid concentration of 5 nM for a given Dmc1-ssDNA filament amount, with increasing concentrations of ScHop2-Mnd1 (0, 0.125, 0.5, 1, 2, and 4  $\mu\text{M}$ ). *D*, optimal amount of ScHop2-Mnd1 titrates with dsDNA target. Three different concentrations of dsDNA plasmid (1.25, 2.5, and 5 nM) were assayed. For each dsDNA concentration, D-loop activity was determined with a fixed amount of Dmc1-ssDNA filaments preassembled with 40 nM ssDNA and Dmc1, with increasing concentrations of ScHop2-Mnd1 (0, 0.125, 0.5, 1, 2, and 4  $\mu\text{M}$ ).

Taken together, these results indicate that ScHop2-Mnd1 has to first interact with dsDNA to fully exert its stimulatory effect on ScDmc1-ssDNA filament. The results are also consistent with the observed  $\text{Ca}^{2+}$  dependence of the reaction in suggesting that the main stimulatory effect of ScHop2-Mnd1 on ScDmc1 occurs after filament formation, during the homology search and/or strand invasion steps of strand exchange in homologous recombination.

**ScHop2-Mnd1 Does Not Stimulate ScRad51-mediated Strand Exchange**—Mammalian Hop2-Mnd1 has been shown to stimulate the D-loop and strand exchange activities of mouse and human Dmc1 and Rad51 (15, 30). However, we found that ScHop2-Mnd1 could not stimulate ScRad51-mediated strand exchange in D-loop assays (Fig. 6A). No stimulation of ScRad51 by ScHop2-Mnd1 was observed over the time course of a D-loop reaction, despite strong stimulation of ScDmc1 activity in parallel experiments (Fig. 6B). This specific stimulatory effect by ScHop2-Mnd1 of Dmc1, but not of Rad51, is different from its mammalian counterpart, but similar to that in fission yeast Hop2-Mnd1 (8).



**FIGURE 6. ScHop2-Mnd1 does not stimulate ScRad51-mediated strand exchange.** *A*, comparison of D-loop formation mediated by either ScDmc1 (1.5  $\mu\text{M}$ ) or ScRad51 (1.5  $\mu\text{M}$ ) in the presence of ScHop2-Mnd1. The concentrations of ScHop2-Mnd1 were 0.25, 0.5, 1, and 2  $\mu\text{M}$ . The gel image from the D-loop assay is shown. *Error bars* represent S.D.,  $n = 8$ . *B*, time course of D-loop formation. When present, ScHop2-Mnd1 (H2M1) concentration was 1  $\mu\text{M}$  in the reactions. *Error bars* represent S.D.,  $n = 3$ .

Because ScRad51 alone and ScHop2-Mnd1 alone did not show D-loop forming activity (Fig. 7A, lanes 2 and 3), and ScHop2-Mnd1 did not stimulate ScRad51 D-loop formation, a trivial interpretation may have been that the ScRad51 used in these experiments was inactive. To control against this possibility, we took advantage of previous work showing that the recombination protein Rad52 can stimulate Rad51 D-loop activity (45, 46), *i.e.* we tested the D-loop forming ability of ScRad51 in the presence of ScRad52. Indeed, we found ScRad51 was responsive to Rad52 stimulation, resulting in about 12% of dsDNA being incorporated into the D-loops (Fig. 7A, lanes 4–9). Therefore, we concluded that the ScRad51 used in our experiments was active and that ScHop2-Mnd1 does not stimulate ScRad51 under the conditions examined.

Another concern was that ScHop2-Mnd1 might stimulate ScRad51 to form D-loops but with a different metal requirement than Dmc1. However, we found that ScHop2-Mnd1 did not stimulate ScRad51-mediated D-loop activity in the presence of  $\text{Mg}^{2+}$  alone,  $\text{Mg}^{2+}$  plus  $\text{Ca}^{2+}$ , or  $\text{Ca}^{2+}$  alone (Fig. 3, lanes 4 and 5). These results are consistent with previous work showing that ScRad51 alone has no activity in the presence of  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  ions (32).



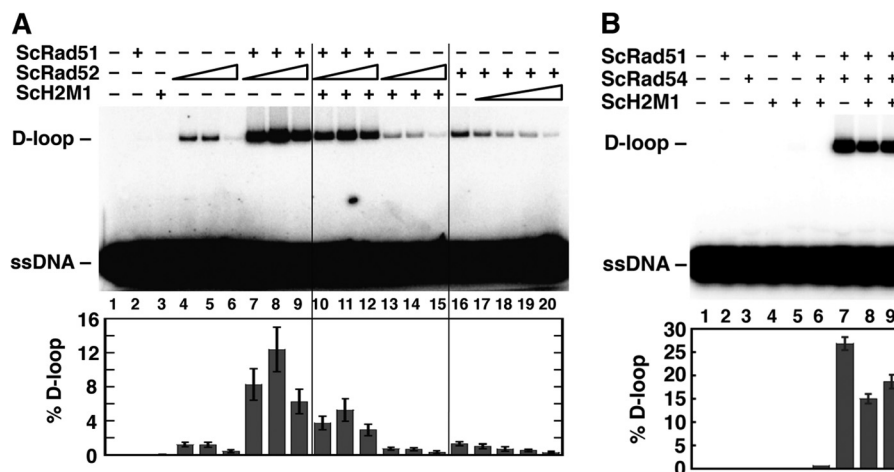


FIGURE 7. **ScHop2-Mnd1 inhibits ScRad51-dependent and ScRad51-independent D-loop formation by ScRad52 and ScRad54.** The gel images from D-loop assays are shown. The results from three replicates were graphed. Error bars represent S.D.,  $n = 3$ . **A**, lanes 1–15, ScRad51 (1.2  $\mu\text{M}$ ), ScRad52 (0.6, 1.2, and 2.4  $\mu\text{M}$ ), and with or without ScHop2-Mnd1 (1  $\mu\text{M}$ ). Lanes 16–20, the ScRad52 (0.6  $\mu\text{M}$ ) D-loop forming activity in the presence of increasing concentrations of ScHop2-Mnd1 (0, 0.5, 1, 1.5, and 2  $\mu\text{M}$ ). **B**, ScRad51 (1.2  $\mu\text{M}$ ), ScRad54 (0.2  $\mu\text{M}$ ), and with or without ScHop2-Mnd1 (1  $\mu\text{M}$  for lanes 4–6 and 8; 1.5  $\mu\text{M}$  for lane 9).

**ScHop2-Mnd1 Inhibits Rad51- and Rad52-mediated D-loop Formation**—Using conditions under which Rad52 stimulates Rad51-mediated D-loop formation, we found that the addition of ScHop2-Mnd1 (1  $\mu\text{M}$ ) to reaction mixtures decreased D-loop yields by about 55% (Fig. 7A, compare lanes 10–12 with 7–9). In addition to its effects on Rad51 D-loop activity, Rad52 has intrinsic, Rad51-independent, D-loop activity that is likely to play a critical role in Rad51-independent recombination *in vivo* (47). We therefore tested whether ScHop2-Mnd1 could suppress Rad52 D-loop activity and found that it could (Fig. 7A, compare lanes 13–15 with 4–6); as the concentration of ScHop2-Mnd1 was increased from 0.5 to 2  $\mu\text{M}$ , D-loops formed by ScRad52 (0.6  $\mu\text{M}$ ) were reduced up to 84% (Fig. 7A, compare lanes 17–20 with 16).

The inhibition by ScHop2-Mnd1 seen above (Fig. 7A, lanes 10–12) could involve either ScRad51 or Rad52. To assess the effect of ScHop2-Mnd1 on Rad51-dependent strand exchange independently of Rad52, Rad54 was used as a Rad51 stimulatory factor (48, 49). We found that ScHop2-Mnd1 inhibits ScRad51 D-loop formation by about 30% (Fig. 7B, compare lanes 8 and 9 with 7). Bovine serum albumin added to the same concentrations as ScHop2-Mnd1 did not lower the yield of D-loops (data not shown), suggesting that this effect was not due to protein crowding. Taken together, these results confirm that ScHop2-Mnd1 can inhibit the D-loop activity of both ScRad51 and ScRad52.

## DISCUSSION

*HOP2* is one of the eight known budding yeast genes that contains more than one intron; these genes are *SUS1*, *VMA9*, *HMRA1*, *YOS1*, *HOP2*, *YGR001C*, *RPS22B*, and *RPL7B* (22). *HOP2* is the only one of these genes known to be involved in meiotic recombination. It remains to be investigated whether splicing plays any role in regulation of *HOP2* expression. Regulated splicing can require specialized factors. In meiosis, the factors known to be involved in regulated splicing are Mer1 and/or Nam8. Two distinct types of meiosis-specific splicing regulation have been described previously. One of these regulatory mechanisms involves the use of a 5' splice site that differs

from the canonical 5' splice site of GUAPyGU. However, the 3' intron of *HOP2* has canonical splice sites, and although the 5' intron has a non-canonical 5' splice site, splicing of this 5' intron was shown previously not to require Mer1 and Nam8 (28). Thus, splicing of both *HOP2* introns may be constitutive. In any event, our data clearly show that both introns are efficiently spliced during wild type meiosis.

We show here that stimulation of Dmc1 D-loop activity by Hop2-Mnd1 is robust. The addition of the accessory factor stimulates the yield of D-loops formed by Dmc1 up to 30-fold. This finding is consistent with *in vivo* analysis of recombination intermediates at meiotic recombination hot spots. Both *mnd1* and *hop2* single mutants display a complete block in conversion of meiotic DSBs to stabilized D-loops (13, 16, 19–21). This phenotype is quite similar to that of a *dmc1* single mutant (4, 50) and indicates that the ability of Dmc1 to promote joint molecule formation *in vivo* is completely Hop2-Mnd1-dependent. The robust stimulation observed for Dmc1 is also consistent with studies of the *Mus musculus* orthologs; the addition of Hop2-Mnd1 converted MmDmc1 from a protein with about 2% D-loop activity to one that promotes up to 70% of input duplex to D-loops, a 35-fold stimulation (15).

We show here that  $\text{Ca}^{2+}$  is required for Hop2-Mnd1-mediated stimulation of Dmc1. Importantly, the addition of  $\text{Mg}^{2+}$  at physiological levels alters the requirement for  $\text{Ca}^{2+}$  such that rather than being required at  $\text{Ca}^{2+}$  concentrations above 250  $\mu\text{M}$ , stimulation of Dmc1 can be observed at  $\text{Ca}^{2+}$  concentrations as low as 0.1  $\mu\text{M}$ . The 1000  $\mu\text{M}$  concentration of  $\text{Ca}^{2+}$  required for maximal stimulation is also reduced by the addition of  $\text{Mg}^{2+}$  to 50  $\mu\text{M}$ . This is a significant finding because it demonstrates that  $\text{Ca}^{2+}$  can activate Dmc1 activity at or near physiological concentration. Furthermore, these findings suggest that, rather than competing with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  enhances the ability of  $\text{Ca}^{2+}$  to promote D-loop formation. It is well documented that the assembly of an active filament requires the binding of a divalent cation and ATP at the ATPase center of the recombinase. Our results are consistent with this interpretation. Furthermore, the ability of  $\text{Mg}^{2+}$  to enhance  $\text{Ca}^{2+}$  activ-

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ity is consistent with the possibility that the two metals simultaneously bind different sites within the same protomer of the active ScDmc1-ssDNA filament. Notably, previous studies of human Dmc1 showed that the concentration of free  $\text{Ca}^{2+}$  rather than the concentration of  $\text{Ca}^{2+}$ -ATP determines the degree to which  $\text{Ca}^{2+}$  stimulates hDmc1, suggesting that  $\text{Ca}^{2+}$  stimulation of Dmc1 involves a different interaction than that seen with  $\text{Mg}^{2+}$  (33). Furthermore, crystallographic data obtained with *Methanococcus voltae* RadA/Rad51, a ScDmc1 homolog, provides a precedent for the possibility that  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  can occupy different sites at the ATP center (51). Two conformations of MvRadA have been observed in crystallized filaments (51, 52). 1) A "loose" conformation, in which DNA-binding loop 2 (L2) is disordered, is observed in the absence of  $\text{Ca}^{2+}$  and resembles an inactive or post-hydrolysis intermediate where the L2 region is not fully engaged in binding DNA (51, 53). 2) A "tight" conformation, in which L1 and L2 DNA-binding loops are largely ordered, is observed in filaments containing  $\text{Mg}^{2+}$  as well as  $\text{Ca}^{2+}$  ions; this form resembles active filaments (51). The implication of these findings is that  $\text{Ca}^{2+}$  promotes an allosteric conformational change in the L2 region to facilitate DNA binding. Thus, the well documented filament stabilization of Dmc1 by  $\text{Ca}^{2+}$  (33, 34) may also involve an analogous allosteric change of loop 2. These considerations led us to hypothesize that  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  bind different sites in the ScDmc1 protomer, thereby promoting a tight nucleoprotein filament that serves as a substrate for Hop2-Mnd1-dependent stimulation. Crystallographic data will be required to test this hypothesis.

The recruitment, loading, and stabilization of Dmc1 presynaptic filaments *in vivo* requires Rad51-Mei5-Sae3; *rad51*, *mei5*, and *sae3* single mutants display defective DSB-dependent assembly of immunostaining foci of Dmc1 (10, 11, 54, 55). In contrast, Hop2 and Mnd1 are *not* required for Dmc1 focus formation (19, 21). Dmc1 foci appear to assemble normally in *hop2* and *mnd1* mutants but remain indefinitely bound to chromosomes in prophase arrested cells. No joint molecules are detected. If the Dmc1 foci formed in *hop2* and *mnd1* mutants represent normal Dmc1 assemblies, then Hop2 and Mnd1 are not essential for the recruitment or stabilization of Dmc1 complexes at sites of recombination *in vivo*. Thus, although not definitive, available cytological data suggest that Hop2-Mnd1-mediated stimulation of Dmc1 occurs at a post-assembly stage. The results of our order-of-addition experiments are consistent with Hop2-Mnd1 functioning after Dmc1 binds ssDNA; pre-binding of Hop2-Mnd1 to the duplex target and Dmc1 to the ssDNA substrate provided the most effective stimulation. Given the well known ability of  $\text{Ca}^{2+}$  to enhance filament formation and stability, the  $\text{Ca}^{2+}$  requirement observed in our experiments also suggests that Hop2-Mnd1 stimulation requires a stable Dmc1 filament. As additional factors that mediate Dmc1 filament formation and stimulate its strand exchange activity, Rad51, Mei5, and Sae3 might augment the function of  $\text{Ca}^{2+}$  in forming substrates for Hop2-Mnd1-stimulated D-loop reactions.

The data described here show clearly that ScHop2-Mnd1 specifically stimulates ScDmc1-mediated, but not ScRad51-mediated, D-loop formation under the various conditions

examined. A substantial body of genetic data in *S. cerevisiae* also supports the hypothesis that Hop2-Mnd1 stimulates Dmc1 but not Rad51 activity *in vivo* (13, 16, 19–21). The strongest support for this claim comes from a recent study involving genetic analysis of the function of the Rad51 inhibitor Hed1 (3). Although normally inhibited during meiosis, Rad51 D-loop activity can be activated by mutation of the *HED1* gene. It is therefore possible to study the requirements for Rad51 D-loop activity in meiosis using a *dmc1 hed1* double mutant background. Importantly, a *dmc1 hed1 mnd1* triple mutant showed a level of recombination activity very similar to that of a *dmc1 hed1* double mutant. This suggests that Rad51 activity in meiosis may not be substantially modulated by Hop2-Mnd1. It is also worth noting in this context that transcription of both the *HOP2* and the *MND1* genes is meiosis-specific in budding yeast, and therefore Hop2-Mnd1 is unlikely to contribute to Rad51 activity during somatic growth (19, 20, 56).

Data from other organisms is consistent with the proposal that Hop2-Mnd1 activity is normally Dmc1-specific during meiosis *in vivo*. In *Schizosaccharomyces pombe*, Mcp7 (Hop2) and Meu13 (Mnd1) expression are restricted to meiosis and *dmc1* is epistatic to more severe mutations in *mcp7* (57, 58). Further, *S. pombe* Mcp7-Meu13 (Hop2-Mnd1) stimulates Dmc1-dependent, but not Rad51-dependent, strand invasion *in vitro* (8). A recent study in *Arabidopsis* provided evidence that Hop2-Mnd1 activity in meiosis is specific to Dmc1 (59); Rad51-mediated recombination is blocked in *mnd1* mutants, but not *dmc1 mnd1* mutants, suggesting that Dmc1 inhibits a Hop2-Mnd1-independent activity of Rad51 in a manner similar to that observed in budding and fission yeast (3, 57, 58). In mice, the *Hop2*<sup>-/-</sup> mutant displays phenotypes that are strikingly similar to those displayed by *Dmc1*<sup>-/-</sup> mutants: accumulation of DSBs, little homologous synapsis, and meiotic arrest (60, 61). Overall, all of the recombination defects described in *Hop2*<sup>-/-</sup> mice can easily be accounted for by the failure of Dmc1-dependent recombination. Of particular importance is the finding that, like *Dmc1*<sup>-/-</sup> mutants, the development of *Hop2*<sup>-/-</sup> mice is normal, and heterozygous crosses demonstrated Mendelian inheritance ratios (60, 61). This is in contrast to *Rad51*<sup>-/-</sup> mice, which die during early embryogenesis (62, 63). Thus, it is possible that the ability of mammalian Hop2-Mnd1 to stimulate Rad51 activity in purified systems (15) may not reflect an essential role in Rad51-mediated, Dmc1-independent recombination. Further studies are required to clarify this important issue.

Not only were we unable to find conditions under which Hop2-Mnd1 stimulates Rad51, but we found that the heterodimer can inhibit that the D-loop activity of Rad51 and Rad52. The biological relevance of this inhibitory activity, if any, remains to be determined. As mentioned above, mutation of the *MND1* gene in the *dmc1 hed1* double mutant background did not alter the level of recombinants, suggesting that Hop2-Mnd1 does not block Rad51 D-loop activity *in vivo*. We are unaware of any relevant data regarding possible inhibition of Rad52 D-loop activity in meiosis.

The experiments presented here indicate that Hop2-Mnd1 functions as an activator of Dmc1-mediated strand exchange; but what is the biological imperative necessitating a role for

Hop2-Mnd1 as a co-factor? One potential answer lies in the fact that Dmc1-mediated meiotic strand exchange occurs preferentially with a distant homologous chromatid rather than the nearby sister chromatid. This meiosis-specific regulation of recombination partner choice is important in yeast to ensure high fidelity reductional chromosome segregation. Hop2-Mnd1 might specifically activate Dmc1 activity in a spatially restricted manner that avoids recombination between sister chromatids. This hypothesis is consistent with previous work showing that Hop2-Mnd1 immunostaining foci do not colocalize with foci of proteins that load directly at sites of DNA double strand breaks (21). We propose that Hop2-Mnd1 is actively precluded from regions near DSB sites but binds randomly to other regions of the genome prior to strand exchange and close chromosome pairing. In effect, Hop2-Mnd1 would be depleted from the target region of the sister chromatid due to the proximity of that region to the DSB. Hop2-Mnd1 bound to the more distant target sequences on the homologous chromatids would promote their invasion. The stage during which stable strand assimilation is activated by Hop2-Mnd1 might be brief relative to the overall time during which Dmc1 and Hop2-Mnd1 are dynamically interacting with DNA, explaining the low frequency of colocalization. These ideas are similar to those proposed by Klein and colleagues (21) but also take into account the more recent findings that Hop2-Mnd1 stimulates Dmc1 activity. Further studies will be required to test the predictions of the “DSB-proximal depletion” model for regulation of Dmc1 by Hop2-Mnd1.

In summary, the previously studied version of Hop2-Mnd1 was only partially functional. The fully functional heterodimer stimulates robust D-loop formation, dependent on physiological concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . These findings set the stage for further biochemical reconstitution of regulated meiotic recombination reactions. Our finding that the stimulatory activity of budding yeast Hop2-Mnd1 is specific for Dmc1-mediated D-loop formation in a purified system is consistent with genetic observations made in diverse organisms. Together, these results suggest that regulation by Hop2-Mnd1 may underlie a key component of the functional specialization of Dmc1 and Rad51 during meiotic recombination.

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