The mouse Hop2 and Mnd1 proteins, which can form a stable heterodimeric complex, ensure the proper synapsis of homologous chromosomes in meiosis by acting in concert with Rad51 and Dmc1 to promote the strand invasion (D-loop formation) step of homologous recombination. Hop2 alone promotes D-loop formation, but Mnd1 and the Hop2-Mnd1 complex do not. Here we show that only the heterodimer complex, but not the individual proteins, can stimulate strand invasion by Dmc1. Furthermore, we demonstrate that the interaction with Mnd1 provokes changes in Hop2 that are responsible not only for abrogating the recombinase activity of Hop2 but also for generating a new molecular interface able to physically interact with and stimulate Dmc1. We also show that coiled-coil motifs in Hop2 and Mnd1 are essential for their interaction with each other and that a clearly delineated region near the COOH terminus of both proteins is necessary for both the DNA binding and single-strand annealing by the Hop-Mnd1 heterodimer. Finally, we describe a point mutation in Hop2 that dissociates its strand invasion activity from its ability to bind and anneal DNA.

During meiosis, diploid precursor cells produce haploid gametes as two rounds of chromosome segregation (meiosis I and meiosis II) follow a single round of DNA replication. In the cell division of meiosis I a high level of recombination occurs between homologous chromosomes. This recombination is required for the development of chiasma, which physically connect homologous chromosomes and ensure the proper chromosome segregation in most organisms.

Homologous recombination is initiated by double-strand breaks (DSBs) introduced by Spo11, a type II-like topoisomerase protein (1–4). The DSBs are degraded from their 5′-ends to expose single-stranded tails with 3′ termini. These processed DNA ends invade homologous double-stranded DNA in non-sister chromatids resulting in the formation of joint molecules. This step of strand invasion is the first stable interaction in homologous recombination. In eukaryotes, two homologues of RecA, Dmc1 and Rad51, are known to catalyze efficient invasion and strand exchange in vitro (5–14).

Several studies (15–21) implicate Hop2 and Mnd1 in meiotic homologous recombination. A Saccharomyces cerevisiae hop2 deletion mutant fails to sporulate due to a uniform arrest at the pachytene stage of meiosis I with the chromosomes engaged in synapsis with non-homologous partners, suggesting a defect in a Hop2-dependent step during meiotic recombination (15, 16). In addition, hop2 knock-out mouse spermatocytes show meiotic arrest and limited chromosome synapsis, consistent with a failure in recombination (17). The MND1 gene was first described in a screen for genes with meiotic specific expression (18), and the S. cerevisiae null mutant strain shows that cells initiate recombination but do not form heteroduplex DNA or a double Holliday junction suggesting that Mnd1 is probably involved in strand invasion (19). Accordingly, results obtained with the mcp7 null mutant, a Schizosaccharomyces pombe ortholog of Mnd1, showed that this strain arrests in meiotic prophase with a reduction in recombination rates and spore viability, a phenotype similar to the one described for meu13 (a Hop2 ortholog) (21). In agreement with these results, biochemical data show that Hop2 and Mnd1 work together to stimulate strand assimilation mediated by Dmc1 and Rad51 (22, 23), suggesting a central role for Hop2 and Mnd1 in the homologous recombination process.

In this work we present a biochemical and biophysical study on Hop2, Mnd1, and Hop2-Mnd1 important to understand the role of these proteins during meiotic homologous recombination. We find that the interaction of Mnd1 with Hop2 leads to changes in the biochemical properties and oligomerization state of Hop2 and an accompanying negative regulation of the Hop2 recombinational activity. Interestingly, these changes suggest that the formation of the Hop2-Mnd1 heterodimer results in a new interface that is responsible for the interaction and stimulation of the Dmc1 recombinase. Furthermore, we have carried out a mutational study of Hop2 and Hop2-Mnd1 that dissects some of the structural/functional requisites for these proteins.

**EXPERIMENTAL PROCEDURES**

Purification and Refolding of Recombinant Mnd1—Murine MND1 was cloned in pET-15b (Novagen) at the Ndel-Xhol sites to generate a protein linked to a histidine tag on the amino terminus. The protein was overexpressed in *Escherichia coli* BL21(DE3). The cells were grown to an *A*<sub>600</sub> = 0.6–0.8, and Mnd1 synthesis was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside for 2 h at 37 °C. Cells were then harvested by centrifugation, washed in chilled buffer (20 mM HEPES (pH 8.0), 100 mM NaCl, 5 mM EDTA), resuspended in lysis buffer (20 mM HEPES (pH 8.0), 100 mM NaCl, 0.5% Triton, 5 mM EDTA, lysosome 200 μg/ml, protease inhibitors (Roche Applied Science), 0.4 unit/ml DNase I) and lysed by sonication. Inclusion bodies were collected by centrifugation at 18,000 × g at 4 °C and washed in lysis buffer without EDTA and lysosome and once in the following buffer: 20 mM HEPES (pH 8.0), 10% glycerol, 0.5 mM NaCl, and protease inhibitors. Next, inclusion bodies were solubilized overnight with agitation in 50 mM HEPES (pH 8.0), 0.5 mM NaCl, 10% glycerol, and 1.8 mM guanidine hydrochloride (GdnHCl). Solubilized inclusion bodies were centrifuged 30 min at 18,000 × g, the supernatant was diluted 20–40 times in refolding buffer.
solution (500 mM NaCl, 50 mM HEPES (pH 8.0), 10% glycerol), and the refolding was allowed to proceed during 15 h at 4 °C. The solution containing the refolded protein was centrifuged 30 min at 8,000 × g and loaded on a nickel-nitrotriacetic acid column previously equilibrated with refolding buffer. The column was washed with 5 volumes of refolding buffer, and bound protein was eluted using a 5–500 mM imidazole linear gradient. The peak corresponding to Mnd1 was loaded on hydroxypatite (Bio-Gel HT Gel, Bio-Rad) and washed with 10 column volumes of washing buffer (300 mM NaCl, 50 mM HEPES (pH 8.0), glycerol 10%). The protein was then eluted with a 100-mL linear gradient (0–300 mM) of phosphate buffer prepared in washing buffer. Finally, Mnd1 was concentrated by ultrafiltration (centriprep YM-10, Amicon) and loaded on Superdex 200 (HP16/60, GE Healthcare) in 50 mM HEPES (pH 8.0), 300 mM NaCl and 10% glycerol. Fractions corresponding to the protein peak were pooled, concentrated using Centriprep YM-10 (Millipore), and stored in aliquots at −80 °C.

**Expression and Purification of Recombinant Hop2 and the Hop2-Mnd1 Complex**—The mouse Hop2 and the Hop2-Mnd1 complex were purified essentially as described previously (23). Briefly, HOP2 or Hop2 and MND1 together were cloned in a pET-15b vector (Novagen) to generate a protein linked to a histidine tag on the amino terminus (only Hop2 was His tagged in the Hop2-Mnd1 complex). The proteins were overexpressed in *E. coli* BL21 (DE3) and purified using the following series of chromatographic steps: nickel-nitrotriacetic acid–agarose (Qiagen), hydroxypatite (Bio-Gel HT Gel, Bio-Rad), Mono Q, and Superdex 200. The proteins were concentrated and stored in buffer (20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% glycerol) at −80 °C.

**Construction and Purification of the Hop2-Mnd1 Complex Deletion Mutants**—The Hop2-Mnd1 complex mutants containing COOH-terminal deletions in Hop2 and Mnd1 were constructed with a site-directed mutagenesis kit (QuickChange XL, Stratagene) by replacing a specific codon of the cDNA for a stop codon. We used the pET-15b plasmid containing both HOP2 and MND1 cDNA (see above) (or pET-15b containing only HOP2 cDNA, for Hop2 mutants) for the generation and expression of the following COOH-terminal Hop2-Mnd1 mutants: ΔC28Hop2, ΔC36Hop2, ΔC55Hop2, ΔC67Hop2, ΔC74Hop2, and ΔC92Hop2, which lack 28, 36, 55, 67, 74, and 92 amino acids from the Hop2 COOH-terminal end, respectively, and ΔC20Mnd1, ΔC55Mnd1, ΔC77Mnd1, ΔC92Mnd1, and ΔC106Mnd1, which lack 20, 55, 77, 92, and 106 amino acids from the Mnd1 COOH-terminal end, respectively. We used the same approach to generate the Hop2-Mnd1 complex mutants in which Met-110 and Gliu-136 from the Hop2 sequence were changed to Ala (Met-110Mnd1, Gliu-136Mnd1, Met-110Mnd1/Gliu-136Mnd1, Met-110Mnd1/Gliu-136Mnd1, and Met-110Mnd1/Gliu-136Mnd1). These mutants were expressed and purified essentially as described above (9). A 12-mer oligonucleotide described above (oligonucleotide 5) was used to generate the C92Hop2, which lack 28, 36, 55, 67, 74, and 92 amino acids from the Hop2 sequence. The C84Mnd1, C92Mnd1, and C106Mnd1, which lack 20, 55, 77, 92, and 106 amino acids from the Mnd1 sequence, were also generated using the same approach.

**Sucrose Density Gradient Centrifugation**—The centrifugation was performed using a TLS55 rotor in an Optima TLX ultracentrifuge (Beckman) in 4 to 20% linear sucrose gradients (2 ml) prepared in 20 mM Tris-HCl (pH 7.4) and 300 mM NaCl. Samples volume was 10 μl and centrifuged at 45,000 rpm at 4 °C for 14 h. The following proteins with known sedimentation coefficients were used to generate a calibration curve: cytochrome c (1.8 S; 12.4 kDa), bovine serum albumin (4.3 S; 66 kDa), alcohol dehydrogenase (7.6 S; 150 kDa), and catalase (11.3 S; 232 kDa). Subsequently, the gradient was fractioned and analyzed by SDS-PAGE.

**DNA Binding Assay**—φX174 circular ssDNA (virion) (30 μM nucleotides), φX174 supercoiled dsDNA (RFI form), nicked dsDNA φX174 (RFII form), or φX174 linear dsDNA (RFI form Xhol-digested) (15 μM bp) was mixed with Hop2, Mnd1, or Hop2-Mnd1 complex in 30 μl of 20 mM Tris-HCl buffer (pH 7.4), 100 mM NaCl, 10% glycerol, and 1 mM MgCl2. The reaction mixtures were incubated at 37 °C for 10 min, and products were resolved at 4 °C in 1% agarose gel electrophoresis in 1× TAE buffer (40 μM Tris acetate (pH 8) and 1 mM EDTA) run at 9 V/cm for 1.5 h. Finally, the bands were visualized by ethidium bromide stain.
ing. When oligonucleotides were used (#3 and #4, 60-mer oligonucleotides and #6 and #7, 100-mer oligonucleotides) the protein was incubated with 25 μM (nucleotides) of 32P-labeled DNA in the following buffer: 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 2 mM MgCl2, and 100 mM NaCl in a volume of 20 μl for 10 min at 37 °C. The samples were mixed with 3 μl of loading buffer (30% sucrose, 0.1% bromphenol blue) and analyzed by electrophoresis in 8% polyacrylamide gels in 1× TAE buffer at 5 V/cm for 5 h. The formation of nucleoprotein complexes was quantitated using a BAS 2500 Bio-imaging Analysis System (Fuji Medical System).

DNA Annealing Assay—Annealing of 32P-labeled 100-bp oligonucleotide (#6) and M13 mp18 (+ strand) was performed in a 25-μl reaction volume in the following buffer: 20 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM DTT and kept at 37 °C during 10 min. The mixture was then treated by addition of stop buffer (1% SDS, 20 mM EDTA, and 1 mg/ml proteinase K) and incubated during 20 min at 37 °C. Products were separated on 1% agarose gels.

D-loop Formation—The supercoiled pUC18 plasmid (15 μM bp) and a homologous radiolabeled 57-mer oligonucleotide (3 μM nucleotides) (oligonucleotide #5, see “Experimental Procedures”) were mixed in 20 μl of reaction buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2.5 mM MgCl2, and 50 mM DTT (1 mM for Hop2-Mnd1/Dmc1) and incubated at 37 °C for 30 min. The reactions were stopped by the addition of SDS (0.5%) and proteinase K (1 mg/ml) followed by 15 min of incubation at 37 °C. Finally, the products were resolved on 1% agarose and analyzed with a BAS 2500 Bio-imaging Analysis System (Fuji Medical System). The amount of D-loop relative to dsDNA was calculated using Equation 1,

\[
\% \text{ dsDNA in D-loop} = A \times 100 \times \left( \frac{P_{\text{prod}}}{P_{\text{total}}} \right) \quad (\text{Eq. 1})
\]

where \( P_{\text{prod}} \) are pixels per inch of oligonucleotide ssDNA in supercoiled dsDNA (plasmid), \( P_{\text{total}} \) are the sum of pixels per inch of free oligonucleotide ssDNA and oligonucleotide ssDNA in supercoiled dsDNA, and \( A \) is the molar ratio of oligonucleotide ssDNA with respect to dsDNA in the reaction mixture.

Surface Plasmon Resonance—Experiments were done on a BIAcore 3000 instrument at 25 °C. The running buffer was 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl2, 1 mM DTT, and 0.005% surfactant P-20. Dmc1 was bound to one flow cell of a CM5 sensor chip, yielding 1500 response units, using the amine coupling kit provided by BIAcore and a additional flow cell was treated in the presence of bovine serum albumin at 1 mg/ml with a final yield of Hop2, Mnd1, and Hop2-Mnd1 complex was determined using UV absorbance at 280 nm (\( \epsilon_{\text{Hop2}} \), 15,930; \( \epsilon_{\text{Mnd1}} \), 22,190; \( \epsilon_{\text{Hop2-Mnd1}} \), 19,060) (Vector NTI Suite, InforMax).

RESULTS

Purification of Mouse Mnd1 Protein—To characterize the mouse Mnd1 protein, we expressed Mnd1 as a fusion protein with an Nterminal hexahistidine tag (His6 tag). Since our attempt to express the protein in the E. coli BL21(DE3) strain led to a maximal recovery of less than 10% of Mnd1 in the soluble fraction and the protein was extensively degraded, we refolded Mnd1 from inclusion bodies. The protein was solubilized using 1.8 M GdnHCl, refolded, and further purified as described under “Experimental Procedures” (Fig. 1A, lane 6). The identity of the protein was confirmed by mass spectrometry (result not shown) and by Western blot using a polyclonal mouse antiserum raised against Mnd1 (Fig. 1A, lanes 7 and 8). The CD spectrum of Mnd1 shows a characteristic α-helical signature with minima at 208 and 222 nm (Fig. 1B). Denaturation of refolded Mnd1 with 1.8 and 5M GdnHCl results in a loss of the minima at 208 and 222 nm, indicating disruption of α-helical structures acquired during the refolding process (Fig. 1B).

In vitro activity assays, such as DNA binding and DNA annealing demonstrating that the refolded protein is active (Figs. 3, A and B, and 4F, A and B). Most importantly, Mnd1 is able to interact with Hop2 to form a Hop2-Mnd1 complex that can stimulate Dmc1 strand assimilation activity (data not shown). Data obtained from the biophysical characterization of the pro-
tein showed that the refolded Mnd1 behaves as a monodisperse protein (see below).

**Oligomerization of Hop2, Mnd1, and Hop2-Mnd1 Free in Solution**—
We determined the oligomeric composition of Hop2, Mnd1, and the Hop2-Mnd1 complex using chemical cross-linking, gel filtration, and sedimentation in linear sucrose gradients (Fig. 2 and Table 1). The results show that Hop2 and Mnd1 change their oligomerization state to form the Hop2-Mnd1 complex. We observed that Hop2 forms dimers and tetramers and is strongly affected by protein concentration and ionic strength, Mnd1 for a wide range of concentration behaves as a monomeric protein, and the Hop2-Mnd1 complex forms a stable 1:1 heterodimer (similar results were obtained either by co-expressing Hop2 and Mnd1 or by combining the purified proteins). We have used the values obtained by sedimentation in sucrose gradients and gel filtration to estimate a molecular mass in the range of 55,509–65,013 for Hop2 (dimer), 28,633–36,307 for Mnd1, and 44,992–89,125 for the Hop2-Mnd1 complex. Considering that size chromatography can be strongly affected by the asymmetry of the particle, the higher than expected molecular mass for Hop2-Mnd1 (calculated molecular mass based in primary sequence: 52,980 Da) might indicate an elongated shape for the complex.

Next, we used analytical ultracentrifugation to examine the oligomeric structure of the proteins independent of macromolecular shape. We observed that Hop2 behaves mainly as a mix of dimers and tetramers, Mnd1 has a mass corresponding to that of a monomer, and the Hop2-Mnd1 complex showed a molecular mass corresponding to that of a heterodimer (53.6 kDa) (supplemental Fig. 1).

Our biochemical characterization (see below) has been carried out at a range of protein concentrations corresponding to dimer for Hop2, monomer for Mnd1, and heterodimer for the Hop2-Mnd1 complex, indicating that these oligomerization states are the active forms of the proteins.

**The Hop2-Mnd1 Complex Sediments as a Heterodimer When Bound to ssDNA or dsDNA Molecule**—We then studied the oligomerization state of the Hop2-Mnd1 complex in the presence of DNA. Sedimentation equilibrium data collected for single- and double-stranded 60-mer oligonucleotide DNAs are consistent with the presence of a single ideal solute, corresponding to monomeric species for each DNA. Sedimentation equilibrium stud-
ies on a 1:4 stoichiometric mixture of 60-mer ssDNA to Hop2-Mnd1 complex were consistent with the presence of more than one species. Analysis in terms of two single ideal solutes results in free ssDNA (56%) and a 1:1 ssDNA/heterodimer (44%). We observed an additional large mass aggregate (3:3 DNA/protein) present at less than 0.03% of the total material (supplemental Fig. 2B). We note that purification of the ssDNA/Hop2-Mnd1 complex by gel filtration results in a monodisperse solute having a molecular mass consistent with a 1:1 ssDNA/heterodimer complex. Next, we examined a 1:4 stoichiometric mixture of 60-mer dsDNA/Hop2-Mnd1 complex. The results showed that the low mass species observed corresponds to the 1:1 dsDNA/heterodimer complex. Data analysis, in terms of two non-interacting ideal solutes in which the buoyant mass of one of the species, was set at that expected for the 1:1 complex returns a buoyant mass of 88,300 Da for the higher mass species. This value is 2.96 times the buoyant mass of the 1:1 DNA/protein complex, hence the data appear to be consistent with the formation of both 1:1 and 3:3 dsDNA to Hop2-Mnd1 complexes. The 3:3 complex represents about 10% of the total material on a concentration scale (supplemental Fig. 2B).

Since no changes in the association of Hop2-Mnd1 when bound to DNA were detected, we propose that a heterodimeric Hop2-Mnd1 is the active conformation involved in Dmc1 stimulation.

**Comparative Analysis of DNA Binding Activity of Hop2, Mnd1, and the Hop2-Mnd1 Complex**—A prerequisite to further understand the mechanism of the Hop2-Mnd1 action is to know the DNA binding properties of this complex as well as those of the purified Hop2 and Mnd1 proteins. There have been previous reports of extensive localization of yeast Hop2 and Mnd1 to chromosomes (15, 20). In an attempt to reproduce the *in vivo* condition, we analyzed the DNA binding activity of the purified proteins using long DNA substrates (φX174 ssDNA and dsDNA). We observed that Hop2 and Mnd1 have a slightly higher affinity for single-stranded DNA, while the Hop2-Mnd1 complex binds both ssDNA and dsDNA with equal affinity (Fig. 3, A and B).

After DSBs are formed, Spo11 is released from the DSB sites creating an initial 12–26 ssDNA gap (24). Next, an exonuclease activity continues resecting the dsDNA, generating longer ssDNA 3’ tails (25–27). At present it is not clear which of these ssDNA substrates are the initial sites for Dmc1 and Rad51 nucleoprotein filament formation. Since the Hop2-Mnd1 complex works in concert with Dmc1 and Rad51 at these sites, we further characterized the DNA binding activity of Hop2-Mnd1 using short DNA substrates. For this purpose, Hop2-Mnd1 was challenged with both single- and double-stranded 60- and 100-mer oligonucleotide DNAs. As shown in Fig. 3, C and D, in contrast to that observed with long DNA substrates (φX174 DNA) (Fig. 3B), the Hop2-Mnd1 complex shows a clear increased affinity for double-stranded DNA. When 20-mer ssDNA or 20-mer dsDNA were used we detected only 20% of ssDNA substrate bound at 25 μM protein and a $K_{dsDNA}$ of 1.3 μM (results not shown).

Collectively, we show that the DNA binding affinity of Hop2-Mnd1 is dependent on the length of the DNA, with a clear preference for dsDNA when shorter DNA substrates are used.

It is possible that the Hop2-Mnd1 heterodimer bound to DNA ends stimulates Dmc1 strand assimilation activity by inducing the nucleation of the recombinase on DNA. Indeed, a previous report suggested that Hop2-Mnd1 from yeast preferentially binds to the ends of DNA (22). We tested whether this observation could be extended to the mouse Hop2-Mnd1 complex. We carried out gel shift experiments in which the protein was incubated with φX174 nicked dsDNA in the presence of a molar excess of either ssDNA or dsDNA 60-mer oligonucleotide. A supercoiled pUC18 plasmid was used as a competitor DNA without ends. The results obtained show that the shift of φX174 DNA is not affected by the presence of either ssDNA or dsDNA oligonucleotides, suggesting that the murine Hop2-Mnd1 complex does not preferentially bind DNA ends (supplemental Fig. 3). No differences were found when linear φX174 was used (data not shown).

**Mnd1 Suppresses the D-loop Formation Activity of Hop2**—We tested purified Hop2, Mnd1, and Hop2-Mnd1 for their abilities to anneal complementary single-strand DNA molecules. We detected maximum yield of product at 0.58 μM for Hop2, 1.5 μM for Mnd1, and 1.48 μM for the Hop2-Mnd1 complex (Fig. 4, A and B). Therefore, all these proteins have an associated single-strand DNA annealing activity with Hop2 displaying a slightly stronger activity.

We next compared the D-loop formation activities of Hop2, Mnd1, and Hop2-Mnd1 by exploring their ability to catalyze the assimilation of a 57-mer homologous oligonucleotide into a pUC18 supercoiled duplex. Hop2 (4.65 μM) showed the strongest activity with 30.3% product formed in 30 min (a value comparable with the 35% for D-loop formation obtained for RecA under the same conditions), whereas a trace activity was detected for the Hop2-Mnd1 complex (4.65 μM) (1.43% product formed) and Mnd1 (4.65 μM) (maximum yield 2.14%) (Fig. 4I, A and B).

**The Hop2-Mnd1 Complex, but Not the Isolated Mnd1 or Hop2 Proteins, Stimulates Dmc1 D-loop Formation**—We have observed that when incorporated in the Hop2-Mnd1 complex, Hop2 and Mnd1 change their biochemical and biophysical properties. We asked whether these differences are reflected in the ability of the Hop2-Mnd1 complex and the individual proteins to stimulate Dmc1 D-loop formation. As we have shown previously, the addition of the Hop2-Mnd1 heterodimer to Dmc1 results in the maximum conversion of dsDNA substrate into D-loops (68%) (Fig. 5A) (23). In contrast to what has been previously published, we observed no stimulation of Dmc1 by Hop2 (28), whereas
Mnd1 showed a marginal increase in the final efficiency of D-loop formation (1:4 Hop2, Mnd1, and Hop2-Mnd1/Dmc1) (Fig. 5A). We also assayed different ratios of either the Hop2-Mnd1 complex or the isolated proteins (data not shown). At a higher Hop2-Mnd1/Dmc1 ratio (2:1), a decrease in the stimulation of Dmc1 was observed; however, no significant changes were detected as the ratio of either Hop2 or Mnd1 to Dmc1 was altered. We next evaluated the physical interaction between these proteins and conclude that the strength of their binding to Dmc1 correlates with their stimulatory effect on Dmc1-promoted strand assimilation. That is, we observed the strongest binding to Dmc1 by the heterodimer and significantly less binding to Dmc1 by Hop2 or Mnd1 (Fig. 5B).

The Hop2 and Mnd1 Interaction Is Mediated by a Coiled-coil Motif Present in Both Proteins—Primary sequence analysis of Hop2 (29–31) indicates the presence of a coiled-coil region between residues 126 and 155 and a putative leucine zipper domain predicted between residues 84 and 124 (Fig. 6A and supplemental Fig. 4IA and IB) (32). In addition, the amino acid sequence of Mnd1 shows a characteristic coiled-coil region (21) between residues 84 and 146, but no leucine zipper is detected (Fig. 6A and supplemental Fig. 4IC, A and B). The formation and location of these coiled-coil motifs are highly conserved from yeast to human (supplemental Fig. 4IA and Fig. 4IC).

We tested the ability of the mutant proteins to heterodimerize with wild-type protein, co-expressing both wild-type and mutant His6-tagged Hop2 or native Mnd1 in E. coli and subjecting the protein-soluble fraction to affinity chromatography (see supplemental data for details).

The disruption of 28 amino acids from COOH terminus of Hop2 did not alter the heterodimer formation and no substantial changes in protein-protein interaction were observed for either the ΔC36Hop2 or ΔC55Hop2 mutant proteins. In contrast, the truncation of either 67 or 74 residues from the COOH terminus results in inefficient Hop2-Mnd1 complex formation. Finally, the deletion mutant ΔC92Hop2, which removes 96% of the coiled-coil domain, was unable to interact with Mnd1 (Fig. 6A and B, and supplemental Fig. 4IC). We detected a similar level of expression in the soluble fraction for wild-type and Hop2 mutants, which lack 28 or 92 COOH-terminal residues, suggesting that the altered Hop2-Mnd1 complex stability is not a consequence of protein instability (data not shown). In aggregate, these results show that the 55-amino acid residues at the COOH-terminal end of Hop2 are not involved in the formation of the Hop2-Mnd1 complex, and only deletions that include the coiled-coil domain interfere in the association of this protein with Mnd1.

Similar deletions from the COOH terminus of Mnd1 indicate that the 55 amino acid residues at the COOH terminus of Mnd1 are not implicated in heterodimer formation, and only deletions that include the coiled-coil domain interfere in the association of this protein with Hop2 (Fig. 6A and B, and supplemental Fig. 4IC).

We also examined whether changes in single amino acid residues could affect the assembly of the Hop2-Mnd1 complex. We prepared Hop2 mutants in which Met-110 (in the putative leucine zipper motif) was replaced by a Pro residue, and Glu-136 (in the putative coiled-coil region) was substituted by a Pro or an Ala. It is expected that replacement of a residue by a Proline removes structure from a protein domain (not expected for an alanine replacement) and consequently might affect the association between Hop2 and Mnd1. The introduction of the M110P mutation on Hop2 permitted interaction with Mnd1; however, the formation of the Hop2-Mnd1 complex was significantly compromised for the E136P Hop2 mutant but not for the E136A mutant (Fig. 6B).
and supplemental Fig. 4/C). Therefore, modification in the structure of the leucine zipper motif of Hop2 does not affect the interaction between this protein and Mnd1, and the repeating heptads (disrupted by Pro but not by Ala replacement) in the coiled-coil region of Hop2 constitutes the relevant interface between these proteins.

The COOH-terminal Region of Hop2 and Mnd1 Are Required for Efficient Hop2-Mnd1 Complex DNA Binding—The purified Hop2-Mnd1 complex mutants containing COOH-terminal truncations on Hop2 and Mnd1 (supplemental Fig. 5A) were tested for their ability to bind φX174 circular ssDNA. The Hop2-Mnd1 complex containing the ΔC36Hop2 mutation showed the same DNA binding affinity as the wild-type protein. In sharp contrast, Hop2-Mnd1 complexes containing either ΔC55Hop2 or ΔC67Hop2 mutations showed decreased DNA binding, and the ΔC74Hop2 protein results in even more defective Hop2-Mnd1 DNA binding with only 3% of dsDNA shifted at 1 μM protein (Fig. 6B and supplemental Fig. 5B). These results indicate that the Hop2 region from 143 to 180 is required for efficient Hop2-Mnd1 complex binding to DNA.
Similar experiments indicate that the Mnd1 region from 113 to 149 is required for efficient Hop2-Mnd1 complex DNA binding (Fig. 6B and supplemental Fig. 5B).

In aggregate, the results strongly suggest that a clearly delimited zone near the COOH-terminal region of both Hop2 and Mnd1 are involved in the DNA binding activity of the Hop2-Mnd1 complex.

The analysis of Hop2-Mnd1 mutants containing M110P, E136P, and E136A amino acid substitution show that all of these mutant proteins are able to bind DNA at wild-type levels (Fig. 6B). Hence, local structural changes either on the leucine zipper region or on the coiled-coil motif of Hop2 do not affect the DNA binding ability of Hop2-Mnd1.

Not unexpectedly, we detected a decrease in the ssDNA annealing activity for these COOH-terminal Hop2-Mnd1 mutants deficient in DNA binding (Fig. 6B).

Finaly, the COOH-terminal Hop2-Mnd1 mutants deficient in DNA binding showed only a trace D-loop formation activity (data not shown).

An Intact DNA Binding Activity and a Structured Leucine Zipper Domain Are Essential for Hop2 D-loop Formation Activity—In agreement with previously published results (28), a series of deletions from the COOH terminus of Hop2 demonstrates that a region close to the COOH terminus of the protein is responsible for efficient DNA binding. Removing 36 and 55 amino acids results in significantly decreased Hop2 DNA binding, whereas truncation of residues 67 and 74 caused a more deficient DNA binding activity with only 4 and 2% of DNA bound at 1 μM protein, respectively (Fig. 6B and supplemental Fig. 5B). Next, we tested these Hop2 mutants for ssDNA annealing and D-loop formation activity. We observed a negative effect of COOH-terminal truncations on the DNA annealing activity (Fig. 6B). Also, as shown in Fig. 6B, we observed a dramatic drop in D-loop formation for the ΔC36Hop2 mutant and only a trace activity for the Hop2 mutants that lack 55, 67, and 74 COOH-terminal residues. Since DNA binding activity is required for Hop2 D-loop formation and ssDNA annealing activity, the reduced Hop2 activities most likely reflect the deficiency in DNA binding. Most interestingly, we find that a point mutation that removes the structure of the leucine zipper domain of Hop2 (M110P) abolishes its D-loop formation activity. This mutation does not significantly change the DNA binding affinity or the DNA annealing activity of Hop2 (Fig. 6B). On the other hand, mutations compromising the coiled-coil motif of Hop2 (E136P) have no effect on the strand assimilation activity.

DISCUSSION

Biochemical Function of Hop2, Mnd1, and Hop2-Mnd1 during Meiotic Recombination—Previous studies (15–21) implicate Hop2 and Mnd1 in meiotic recombination. However, the biochemical role of the Hop2-Mnd1 complex and the individual proteins remain incompletely understood.
A growing number of observations indicate that Hop2 and Mnd1 work together at the same step during recombination (20, 22, and 23); our results show that, compared with Mnd1 and Hop2-Mnd1, Hop2 has a distinct DNA binding and annealing activity and, more importantly, is a strong recombinase (23). We suggest two possible roles for Hop2, one, which involves its strong intrinsic recombinational activity, and two, a specific stimulatory effect on Dmc1 strand assimilation when Hop2 is incorporated into the Hop2-Mnd1 heterodimer.

The Interaction of Hop2 with Mnd1 Abrogates the Intrinsic Recombinational Activity of Hop2 and Generates a New Protein Interface Able to Interact and Stimulate Dmc1—We showed that the interaction of Hop2 with Mnd1 down-regulates the strand assimilation activity of Hop2 (Fig. 4II and Ref. 23). This result suggests that Mnd1 is a negative regulator of the recombinase activity of Hop2 in vivo. Interestingly, here we observed that Hop2-Mnd1 binds Dmc1 with a significantly increased affinity with respect to the isolated Hop2 and Mnd1, and only the Hop2-Mnd1 heterocomplex, but not the individual proteins, significantly stimulates Dmc1 strand assimilation activity. Thus, the interaction between Hop2 and Mnd1 not only abrogates the intrinsic recombinational activity of Hop2 but also is responsible for revealing a novel function of these proteins, the stimulation of Dmc1 strand assimilation. In addition to these differences in their biochemical properties, we have observed clear differences in the oligomerization state of Hop2, Mnd1, and the Hop2-Mnd1 complex. Taken together, we hypothesize that the exchange of one subunit of the homodimeric Hop2 (or three subunits of the Hop2 tetramer) for an Mnd1 monomer down-regulates Hop2 recombinase activity while generating a novel molecular interface able to stimulate Dmc1 strand assimilation. It is possible that Mnd1 works by inducing conformational changes in Hop2 that unmask the ability of Hop2 to stimulate Dmc1 and/or as a specific physical mediator between Hop2 and the recombinase.

Structural Basis for Hop2 and Hop2-Mnd1 Function—We carried out mutational analyses of Hop2-Mnd1 important to understand the structural/functional requisites of this complex as well as how Hop2 and Mnd1 participate in the formation of an active complex. We find that residues near the COOH-terminal region of Hop2 and Mnd1 are implicated in protein binding to DNA and consequently in single-strand DNA annealing activity. In addition, we demonstrate that the α-helices coiled-coil motif of Hop2 and Mnd1 are essential for stable interaction between these proteins. This motif could be precisely controlled by different factors such as phosphorylation or interaction with ions, making this system a highly versatile structure.

In addition, our data reveals important structural features required for the recombinase activity of Hop2. We observed that the carboxyl-terminal region of the protein is important for efficient DNA binding and essential for its single-strand DNA annealing and D-loop formation activity. Interestingly, we find that a mutation that negatively affects the structure of the leucine zipper domain abolishes the strand assimilation activity of Hop2, even though it does not significantly change the DNA binding and annealing activity of Hop2. Possibly, this domain plays an important role in the coordination of DNA substrates or in the formation of an essential intermediate during strand assimilation.

Our results provide a model for the functional domains of Hop2 and Hop2-Mnd1 that should allow us to target individual amino acids important to DNA binding or protein regions relevant to the interaction of the Hop2-Mnd1 heterocomplex with Dmc1.

Since these proteins are evolutionarily conserved in eukaryotes, the results we have obtained with mouse Hop2 and Mnd1 should be applicable to homologs in other organisms.

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