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Yeast Recombination Factor Rdh54 Functionally Interacts with the Rad51 Recombinase and Catalyzes Rad51 Removal from DNA*

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The *Saccharomyces cerevisiae RDH54*-encoded product, a member of the Swi2/Snf2 protein family, is needed for mitotic and meiotic interhomologue recombination and DNA repair. Previous biochemical studies employing Rdh54 purified from yeast cells have shown DNA-dependent ATP hydrolysis and DNA supercoiling by this protein, indicative of a DNA translocase function. Importantly, Rdh54 physically interacts with the Rad51 recombinase and promotes D-loop formation by the latter. Unfortunately, the low yield of Rdh54 from the yeast expression system has greatly hampered the progress on defining the functional interactions of this Swi2/Snf2-like factor with Rad51. Here we describe an *E. coli* expression system and purification scheme that together provide milligram quantities of nearly homogeneous Rdh54. Using this material, we demonstrate that Rdh54-mediated DNA supercoiling leads to transient DNA strand opening. Furthermore, at the expense of ATP hydrolysis, Rdh54 removes Rad51 from DNA. We furnish evidence that the Rad51 binding domain resides within the N terminus of Rdh54. Accordingly, N-terminal truncation mutants of Rdh54 that fail to bind Rad51 are also impaired for functional interactions with the latter. Interestingly, the *rdh54 K352R* mutation that ablates ATPase activity engenders a DNA repair defect even more severe than that seen in the *rdh54*Δ mutant. These results provide molecular information concerning the role of Rdh54 in homologous recombination and DNA repair, and they also demonstrate the functional significance of Rdh54·Rad51 complex formation. The Rdh54 expression and purification procedures described here should facilitate the functional dissection of this DNA recombination/repair factor.

Homologous recombination (HR)[3] helps eliminate deleterious lesions, including DNA strand breaks and interstrand cross-links, from chromosomes. Furthermore, by linking homologous chromosomes through crossover formation, HR helps ensure the proper segregation of the chromosomes in the first meiotic division. Interestingly, HR can also provide a means of elongating shortened telomeres in the absence of telomerase. Because of its involvement in various aspects of chromosome maintenance, mutants of HR typically accumulate chromosome aberrations and exhibit a mutator phenotype. In fact, in higher eukaryotes, deletion of HR genes often engenders cell inviability, believed to reflect the requirement of HR for cells to successfully complete DNA replication during the S phase (1). That HR is critical for genome stability is underscored by the realization that the cancer-prone diseases Fanconi anemia and Bloom's syndrome exhibit either HR deficiency or HR deregulation, respectively (2, 3). Furthermore, cells deficient in the tumor suppressors BRCA1 and BRCA2 are marked by a pronounced HR defect (4–7). The linkage of HR impairment or deregulation to the cancer phenotype emphasizes the importance of delineating the mechanistic underpinnings of the HR machinery.

The genetic requirement of HR was initially defined in the budding yeast *Saccharomyces cerevisiae*. Studies in this model eukaryote have identified the *RAD52* epistasis group of genes as being needed for mitotic and meiotic recombination and DSB repair by HR (8). The structure and function of the *RAD52* group genes are remarkably conserved among eukaryotes, from yeast to humans (1).

Two *RAD52* group genes, *RAD54* and *RDH54*, encode proteins that belong to the Swi2/Snf2 protein family. As deduced from genetic studies conducted by several research groups, *RAD54* serves a more prominent role than *RDH54* in mitotic DSB repair, intrachromosomal recombination, and sister chromatid-based recombination, whereas *RDH54* is more relevant than *RAD54* in interhomologue recombination in both mitotic and meiotic cells (1, 9–11). Consistent with their Swi2/Snf2 likeness, both Rad54 and Rdh54 proteins possess a DNA-dependent ATPase activity (12, 13). Employing biochemical

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[3] The abbreviations used are: HR, homologous recombination; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NTA, nitrilotriacetic acid; ds, double-stranded.

means and scanning force microscopy, evidence has been presented that Rad54 and Rdh54 use the free energy from ATP hydrolysis to translocate on dsDNA, generating unconstrained negative and positive supercoils in the DNA (13–15). Importantly, the DNA translocase and supercoiling functions appear to be a generally conserved property of Swi2/Snf2 protein family members (16–18).

Yeast two-hybrid studies (19, 20) and biochemical analyses (12, 13) have shown an interaction of Rad54 and Rdh54 with the Rad51 protein, which is structurally related to the *Escherichia coli* RecA protein (21). Like RecA, Rad51 possesses an ATP-dependent recombinase activity that can pair and exchange DNA strands between homologous DNA molecules (22). Interestingly, Rdh54 was found to interact with Dmc1, the meiosis-specific RecA/Rad51-like recombinase enzyme (23, 24), in the yeast two-hybrid system (25). Rdh54 was named Tid1 by the authors of this latter study, to reflect its ability for two-hybrid interaction with Dmc1 (25). The meiotic prominence of Rdh54 (1, 9, 10) could very well be because of its physical and functional interactions with not only Rad51 but with Dmc1 as well.

To understand the molecular function and mechanistic role of the *RDH54* gene in HR and DSB repair reactions, it is necessary to purify its encoded protein and characterize the protein on its own and in conjunction with the Rad51 and Dmc1 recombinases. To facilitate the detailed dissection of Rdh54 biochemical properties and its functional interactions with Rad51, we have devised an *E. coli* protein expression system and a purification protocol that together provide milligram quantities of highly purified Rdh54 and mutant variants of this HR factor. Here we report our biochemical studies that show DNA strand opening by Rdh54 with a dependence on ATP hydrolysis. By expressing, purifying, and characterizing several N-terminally truncated forms of Rdh54, we have been able to ascertain the functional significance of the Rdh54·Rad51 complex. Furthermore, we show that at the expense of ATP hydrolysis, Rdh54 efficiently removes Rad51 from duplex DNA. The ability of Rdh54 to remove Rad51 from DNA is less dependent on the N-terminal Rad51 binding domain than is its D-loop promoting activity. Interestingly, the *rdh54 K352R* mutation that ablates ATPase activity engenders a DNA repair defect even more severe than that seen in the *rdh54*Δ mutant. The biochemical and genetic studies described herein contribute help clarify the role that Rdh54 fulfills in HR, and the protein expression/purification and biochemical systems devised here should facilitate the ongoing functional dissection of Rdh54.

EXPERIMENTAL PROCEDURES

Plasmids—DNA fragments encoding full-length and N-terminally truncated variants of Rdh54 were prepared by the PCR reaction and cloned into the NcoI and XhoI site of the pET32a vector (Novagen) to add thioredoxin and His_6 tags to the N terminus of these proteins. The DNA template for the PCR reaction is pRdh54.1 (13). The forward PCR primers are: 5′-CTATAGGGAGAGCCACCATGGCGGTAATAAGCGTTAAACCTCG-3′ for both full-length Rdh54 and the Rdh54 1–133 polypeptide; 5′-CTATAGGGAGAGCCACCATGGCACAGATACCGAAATATGAG-3′ for the rdh54 Δ34 mutant; 5′-CTATAGGGAGAGCCACCATGGAAAACACTAGATATTTTACTATC-3′ for the Δ102 mutant; 5′-CTATAGGGAGAGCCACCATGGCCAGTAGCGATAAGTTATGC-3′ for the Δ133 mutant. The reverse primers are: 5′-GATCCGCTCGAGTTATCATTGTTCTCTGAGAC-3′ for full-length Rdh54 and the Δ34, Δ102, and Δ133 variants; 5′-GATCCGCTCGAGTTATCATTTTAAGGTAGCGTAGC-3′ for the Rdh54-(1–133) polypeptide. For generating the rdh54 K352R expression plasmid, the Rdh54 expression plasmid was subject to *in vitro* mutagenesis using the QuikChange kit (Stratagene) to alter lysine 352 to arginine (K352R). The primers used for the mutagenesis procedure are: 5′-CCTTTTGGCTGATGATATGGGTTTAGGTCGGACACTAATGAGTATAACTTTGATTTGGACATTAATTAG-3′ and 5′-CTAATTAATGTCCAAATCAAAGTTATACTCATTAGTGTCCGACCTAAACCCATATCATCAGCCAAAAGG-3′. All the protein expression constructs were sequenced to verify that no unwanted mutation had been introduced during the subcloning steps.

Yeast Strains—All the yeast strains used in the genetic experiments were derived from W303 and have the genotype *leu2-3, 112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100 RAD5*.

Homologous Recombination Assays—Recombination rates were determined according to the median method of Lea and Coulson (26) as described previously (27). Fresh zygotes of each indicated genotype were streaked onto solid YPD medium and nine colonies were used for each fluctuation test for rate determinations. Three zygotes were used for each diploid genotype.

DNA Substrates—All the oligonucleotides were obtained from Oligos Etc., Inc. To prepare the DNA substrate for the DNA mobility shift assay, the 80-mer oligonucleotide 1, 5′-TTATATCCTTTACTTTGAATTCTATGTTTAACCTTTTACTTATTTTGTATTAGCCGGATCCTTATTTCAATTATGTTCAT-3′ was 5′ end-labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham Biosciences). Following the removal of the free nucleotide with a spin 30 column (Bio-Rad), the radiolabeled oligonucleotide was annealed to its exact complement by heating the reaction mixture at 85 °C for 3 min and slow cooling to 23 °C. The resulting duplex was purified from a 10% polyacrylamide gel by overnight diffusion at 4 °C into TE (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA). For the D-loop assay, the 90-mer oligonucleotide D1 (14), being complementary to pBluescript SK DNA from positions 1932 to 2022, was 5′ end-labeled and then purified using the MERmaid Spin Kit (Bio101). For the DNA topology modification and DNA strand opening reactions, ϕX174 replicative form I DNA (Invitrogen) was relaxed by treatment with calf thymus topoisomerase I (Invitrogen), as described previously (28).

The 600-bp biotinylated dsDNA used in the experiments in Figs. 5 and 11 was prepared by PCR amplification of pBluescript SK DNA using the 5′-biotinylated primer 1 (5′-AAATCAATCTAAAGTATATATGAG-3′) and non-biotinylated primer 2 (5′-TGAGTACTCACCAGTCACAG-3′). The amplified DNA was deproteinized by phenol-chloroform extraction, ethanol-precipitated, and dissolved in TE. To immobilize the biotinylated dsDNA on streptavidin magnetic beads (Roche Applied Science), 30 μg of the DNA was mixed with 400 μl of beads in 800 μl of buffer A (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and

1 mM EDTA) for 4 h at 25 °C. The beads were washed twice with 800 μl of buffer A containing 1 M NaCl and stored in 400 μl of buffer A at 4 °C. The beads contained 50 ng of the biotinylated DNA per μl of suspended volume. The linear pBluescript SK dsDNA, used as the Rad51 trap in Figs. 5 and 11, was prepared by digestion of replicative form I DNA with the restriction enzyme EcoRV.

Expression and Purification of Full-length Rdh54 and N-terminally Truncated Variants—*E. coli* Rosetta cells (Novagen) harboring plasmids that express either the full-length or N-terminally truncated variants of Rdh54 were grown at 30 °C to A_{600} between 0.6 and 0.8. The culture was shifted to 16 °C and induced with 0.1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 16 h. Cells from 60 liters of culture were harvested by centrifugation and stored at −80 °C. All the subsequent steps were carried out at 4 °C. For protein purification, cells (150 g) were resuspended in 300 ml of buffer B (20 mM KH_2PO_4 pH 7.4, 150 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal (a nonionic detergent purchased from Sigma), 2 mM 2-mercaptoethanol, and the following protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A at 3 μg/ml each, and 1 mM phenylmethylsulfonyl fluoride) and lysed in the French Press. The crude lysate was clarified by ultracentrifugation (60 min at 100,000 × *g*), and the supernatant was applied onto a Q-Sepharose column (2.5 × 24 cm; 40 ml total). The Q column flow was applied onto a SP-Sepharose column (2.5 × 24 cm; 40 ml total). Following a wash with 80 ml of buffer B, the SP-Sepharose column was developed with a 400-ml gradient of 0–325 mM KCl in buffer B. Rdh54 peak fractions were identified by SDS-PAGE and Coomassie Blue staining, pooled, and incubated with 2 ml Ni^{2+}-NTA-agarose (Qiagen) for 2 h at 4 °C. The matrix was poured into a column with an internal diameter of 1.0 cm and then washed with 24 ml of buffer B containing 500 mM KCl, 12 ml each of buffer B containing 10 and 20 mM imidazole, and then with 10 ml of buffer B containing 200 mM imidazole. The 200 mM imidazole eluate was further fractionated in a 0.5-ml Mono S column (Amersham Biosciences), using a 15-ml gradient of 0–325 mM KCl in buffer B. Rdh54-containing fractions were pooled and concentrated to 5 mg/ml in a Centricon-30 microconcentrator (Amicon). The overall yield of highly purified Rdh54 was ~2 mg. The concentrated Rdh54 protein was divided into small aliquots and stored at −80 °C. The truncated Rdh54 variants (Δ34, Δ102, and Δ133) were purified using the same procedure as full-length Rdh54. The Rdh54-(1–133) fragment was purified using the combination of Ni^{2+}-NTA-agarose and Mono S column steps. The concentration of the Rdh54 and rdh54 protein preparations was determined by densitometric scanning of SDS-polyacrylamide gels that contained multiple loadings of proteins against known amounts of bovine serum albumin run in the same gels.

Other Protein Reagents—Rad51and rad51 K191R was purified to near homogeneity from a yeast strain tailored to overexpress this protein, as described previously (29, 30). *E. coli* topoisomerase I was purified to near homogeneity from the *E. coli* strain JM 101 containing plasmid pJW312-sal with the topA gene under the control of the Lac promoter, as described (31).

Pulldown Assay—For affinity pulldown through the His_6 tag on Rdh54 or the truncated variants, Rad51 (4 μg) was incubated with His_6-Rdh54, His_6-Rdh54Δ34, His_6-Rdh54Δ102, His_6-Rdh54Δ133, or the His_6-Rdh54-(1–133) fragment (4.8 μg each) in 30 μl buffer C (20 mM KH_2PO_4, pH 7.4, 75 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM 2-mercaptoethanol) for 30 min on ice. These were incubated with 8 μl of Ni^{2+}-NTA agarose beads for 30 min on ice with gentle mixing every 30 s. The beads were pelleted by centrifugation, and the supernatant was removed. After washing twice with 30 μl of buffer C containing 10 mM imidazole, the beads were treated with 20 μl of 2% SDS to elute bound proteins. The supernatant (8 μl), wash (12 μl), and SDS eluate (8 μl) were subjected to SDS-PAGE to determine their protein contents.

ATPase Assay—The indicated amounts of Rdh54 and truncated variants were incubated at 37 °C in 10 μl of buffer D (35 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 50 mM KCl) containing 100 μg/ml bovine serum albumin, 25 Ci/mol of [γ-^{32}P]ATP at the final concentration of 1 mM, and pBluescript II SK DNA (23 μM base pairs). To examine the effect of Rad51 on Rdh54-mediated ATP hydrolysis, the indicated amount of Rad51 was mixed with pBluescript II SK DNA in buffer D at 37 °C for 3 min prior to the addition of Rdh54 or its truncated variants. Aliquots (2 μl) of the reactions were removed at the indicated times and mixed with an equal volume of 500 mM EDTA to halt the reaction. The level of ATP hydrolysis was determined by thin layer chromatography in polyethyleneimine cellulose sheets (J. T. Baker) with phosphorimaging analysis in a Personal FX phosphorimager using the Quantity One software (Bio-Rad), as described previously (12).

DNA Mobility Shift Assay—The ^{32}P-labeled 80-mer dsDNA (30 nM) was incubated for 5 min at 37 °C with the indicated amounts of Rdh54 and truncated variants in 10 μl of buffer D with or without 2 mM ATP and an ATP-regenerating system consisting of 20 mM creatine phosphate and 30 μg/ml creatine kinase. The reaction mixtures were run in 10% polyacrylamide gels in TAE buffer (40 mM Tris acetate, pH 7.4, 0.5 mM EDTA). The gels were dried onto a sheet of DEAE paper to prevent the loss of the radiolabeled DNA and then subjected to phosphorimaging analysis.

DNA Topology Modification Reaction—The indicated amounts of Rdh54 and truncated variants were incubated for 5 min at 23 °C with topologically relaxed φX174 DNA (10 μM base pairs) in 9.5 μl of buffer D with 2 mM ATP and an ATP-regenerating system, followed by the addition of 100 ng of *E. coli* topoisomerase I. Reaction mixtures (10 μl, final volume) were incubated for 10 min at 37 °C, deproteinized with SDS (0.5%) and proteinase K (0.5 mg/ml) for 3 min at 37 °C, and then analyzed in 0.9% agarose gels run in TAE buffer. The DNA species were stained with ethidium bromide. To examine the effect of Rad51 on the DNA supercoiling reaction, Rdh54 (or one of the rdh54 truncation mutants) was incubated with the indicated amounts of Rad51 in buffer D for 10 min at 23 °C prior to the addition of the DNA substrate and incubation with topoisomerase.

P1 Assay to Monitor DNA Strand Separation—The indicated amounts of Rdh54 and truncated variants were incubated for 2 min at 23 °C with topologically relaxed φX174 DNA (18.5 μM base pairs) in 10 μl buffer D with 2 mM ATP and an ATP-regenerating system, followed by the addition of 0.4 unit of P1

nuclease (Sigma). The reaction mixtures (10 μl, final volume) were incubated for 10 min at 30 °C and then deproteinized with SDS (0.5%) and proteinase K (0.5 mg/ml) for 3 min at 37 °C. The DNA species were resolved in 0.9% agarose gels containing 10 μM ethidium bromide in TAE buffer. To examine the effect of Rad51 on the DNA strand opening reaction, Rdh54 (or one of the rdh54 truncation mutants) was incubated with the indicated amounts of Rad51 in buffer D for 10 min at 23 °C prior to the addition of the DNA substrate and incubation with P1 nuclease.

D-loop Assay—The ^{32}P-labeled 90-mer oligonucleotide substrate (2.4 μM nucleotides) was incubated for 5 min at 37 °C with Rad51 (0.8 μM) in 10.5 μl buffer E (35 mM Tris, pH 7.5, 1 mM dithiothreitol, 5 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, and an ATP-regenerating system). The indicated amounts of Rdh54 and truncated variants were then added in 1 μl, followed by a 1-min incubation at 23 °C. The D-loop reaction was initiated by adding pBluescript replicative form I DNA (35 μM base pairs) in 1 μl. The reaction mixtures were incubated for 5 min at 37 °C, deproteinized, and processed for electrophoresis in 0.9% agarose gels in TAE buffer, as above. The gels were dried onto a sheet of DEAE paper to prevent the loss of DNA and the radiolabeled D-loop was visualized and quantified in the phosphorimager.

Assay to Monitor Rad51 Removal from DNA—To assemble Rad51-dsDNA nucleoprotein filament, Rad51 (3.7 μM) was incubated for 5 min at 37 °C with magnetic beads containing biotinylated dsDNA (15 μM base pairs) in 18 μl of buffer E. After the incorporation of the indicated amounts of Rdh54 or truncated variants in 1 μl and a 3-min incubation at 37°, the reactions were completed by adding linear pBluescript SK dsDNA (75 μM base pairs) in 1 μl. Following a 10-min incubation at 37 °C, the beads were captured with the Magnetic Particle Separator (Roche Applied Science), and the supernatants were set aside. Bound proteins were eluted from the beads with 10 μl of 2% SDS. The various supernatants and SDS eluates (8 μl each) were analyzed by SDS-PAGE and Coomassie Blue staining to determine their content of proteins and also by agarose gel electrophoresis in TAE buffer followed by ethidium bromide staining to reveal the pBluescript dsDNA trap.

RESULTS

Rdh54 ATPase Activity Is Required for Biological Function—Rdh54 possesses an ATPase function that is activated by DNA (13). Our published work (13) and the biochemical studies documented below are consistent with the idea that the Rdh54 ATPase activity is needed for biological efficacy. To obtain genetic evidence to support this premise, we replaced the chromosomal *RDH54* gene with the *rdh54 K352R* allele, whose encoded protein is devoid of ATPase activity (13) (see below). *RDH54* is specifically needed for interhomologue recombination (9–11, 32). Recombination rate determinations of spontaneous mitotic interhomologue recombination between *leu2* alleles showed that, like the *rdh54*Δ mutant, the *rdh54K352R* mutant is recombination deficient (Fig. 1). We next examined haploid and diploid *rdh54K352R* strains for their sensitivity to methyl methanesulfonate (MMS), which induces DNA damage that is repaired by HR.

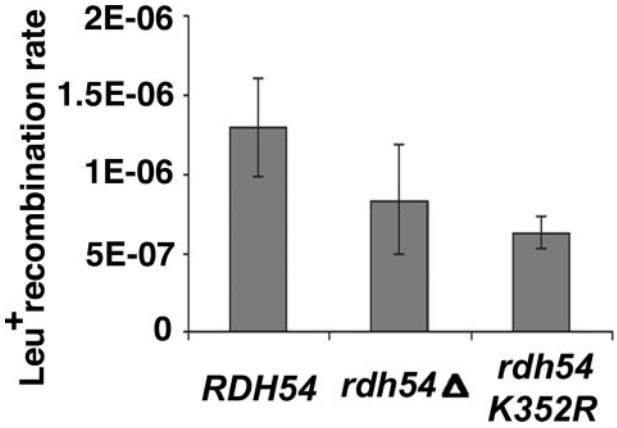

FIGURE 1. **Recombination rates in the wild-type strain and rdh54 mutants.** Rates of recombination between the *leu2-RI* and *leu2-BSTEII* alleles, which are ablations of the EcoRI and BstEII sites within the *LEU2* gene, were determined. Strains used were HKY666–1A and HKY679–2C for *RDH54* (WT), HKY666–3A and HKY679–7D for *rdh54*Δ, and HKY866–1D, and HKY865–4B for *rdh54K352R*.

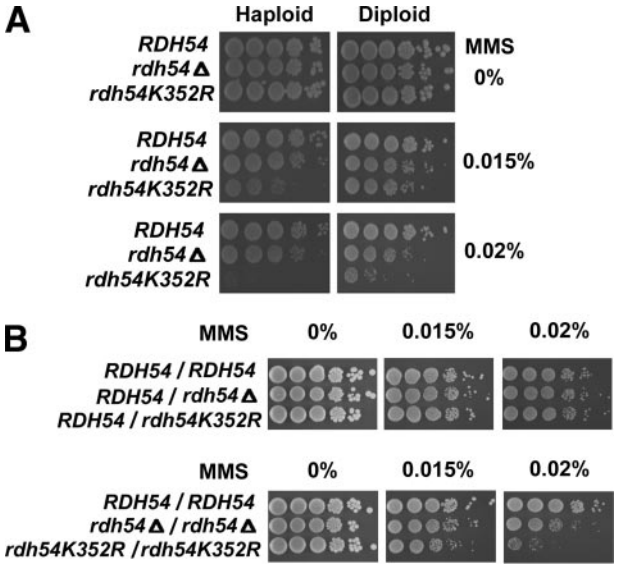

FIGURE 2. **MMS sensitivity of wild-type and *rdh54* mutant haploid and diploid strains.** *A*, MMS sensitivities were examined by plating 5-μl aliquots of 10-fold serial dilutions from freshly grown strains of the indicated genotypes. Prior to dilution, the cultures were adjusted to the same cell concentration. Strains used for haploids were HKY580-10D for *RDH54* (WT), HKY641–5B for *rdh54*Δ, and HKY828 for *rdh54K352R*. Strains used for diploids were HKY580–10A and HKY579–10A for *RDH54* (WT), HKY642–3A and HKY641–5B for *rdh54*Δ, and HKY866–1D and HKY866–1A for *rdh54K352R*. The plates were photographed after 72 h of incubation at 30 °C. *B*, homozygous and heterozygous diploid strains of the indicated genotypes were examined for MMS sensitivity. Strains used for homozygous diploids were HKY579–10A and HKY580–10A for *RDH54/RDH54*, HKY644–1B and HKY644–4B for *rdh54*Δ*/rdh54*Δ, and HKY1832–1B and HKY1832–1C for *rdh54K352R/rdh54K352R*. Strains used for heterozygous diploids were HKY580–10D and HKY644–1B for *RDH54/rdh54*Δ, and HKY580–10D and HKY1832–1B for *RDH54/rdh54K352R*. The plates were photographed after 48 h of incubation at 30 °C.

As shown in Fig. 2*A*, *rdh54K352R* strains are sensitive to MMS, in fact even more so than the *rdh54*Δ strains. The *rdh54K352R* mutation appears to engender a greater increase in MMS sensitivity in the haploid state than in the homozygous diploid state (Fig. 2*A*). We have asked whether the *rdh54K352R* mutation might exert semi-dominance by determining MMS sensitivity in heterozygous diploids, but

TABLE 1

Sporulation efficiency and spore viability as a function of the *RDH54* status

Sporulation efficiency was determined by counting the percentage asci in 200 cells after three days of growth on sporulation medium. Strains used were HKY580-10D and HKY579-10A for *RDH54* (WT), HKY644-4B and HKY644-1B for *rdh54*Δ, HKY1832-1C and HKY1832-1B for *rdh54K352R,* HKY580-10D and HKY644-1B for *RDH54/rdh54*Δ, and HKY580-10D and HKY1832-1B for *RDH54/rdh54K352R.*

Strain	Percentage sporulation	Spore viability (from 100 spores)
	%	%
RDH54/RDH54	47.5	99
*rdh54*Δ*/rdh54*Δ	23.5	73
rdh54K352R/rdh54K352R	20.5	56
*RDH54/rdh54*Δ	51	95
RDH54/rdh54K352R	52	95

found no increase in sensitivity of the heterozygote compared with the homozygous wild-type strain (Fig. 2*B*).

Because *rdh54*Δ diploids are partially impaired for sporulation and show a reduction in spore viability (9), we examined the diploid *rdh54K352R* mutant for sporulation efficiency and spore viability. As summarized in Table 1, the *rdh54K352R* diploid has the same reduced level of sporulation as the homozygous *rdh54*Δ diploid. The *rdh54 K352R* mutation has no dominant effect on sporulation levels in heterozygous diploids (Table 1).

From the above results, we could conclude that ATP hydrolysis by Rdh54 is indispensable for protein functions in both mitotic and meiotic cells. The fact that the *rdh54 K352R* mutation confers increased MMS sensitivity as compared with a deletion of *RDH54* further indicates that the presence of the rdh54 K352R mutant protein is deleterious to DNA repair.

Expression of Rdh54 in E. coli and Its Purification—An in-frame ATG start codon exists 102 nucleotides upstream of the annotated start codon (according to the data base YGD) in the *RDH54* gene. We shall refer to the protein coded by the reading frame that utilizes the upstream ATG as Rdh54 and the shorter protein (as annotated by YGD) rdh54 Δ34, so as to reflect the omission of 34 amino acid residues in the latter. The yeast Rdh54 overexpression system and a multi-step purification protocol that we previously devised yields between 100 and 200 μg of purified Rdh54 protein from 1 kg of yeast paste, being equivalent to 300 liters of yeast culture. Because of the extremely low expression level and the susceptibility of Rdh54 to proteolysis during purification, the purity of the final product is quite variable. These constraints have imposed a great limitation on our ability to conduct biochemical studies on Rdh54. We therefore explored the feasibility of Rdh54 protein production in *E. coli*. For this purpose, thioredoxin and His_6 tags were added to the N terminus of the Rdh54 protein to enhance its solubility and to facilitate its purification of by using nickel-NTA agarose, respectively. The T7 promoter used for Rdh54 expression is IPTG inducible, and extracts made from cells grown in medium containing IPTG harbored a protein species of 125 kDa not found in extracts of either un-induced cells (Fig. 3*A*) or cells harboring the empty protein expression vector grown under inducing conditions (data not shown). The size of the novel protein species is in excellent agreement with the theoretical value of 125 kDa for the tagged Rdh54 protein. That this protein species corresponds to the Rdh54 protein was verified by immunoblot analysis using either anti-histidine antibodies (Fig. 3*A*) or affinity-purified anti-Rdh54 antibodies (13). The Rdh54 protein thus expressed is soluble, and a procedure (Fig. 3*B*) entailing affinity chromatography on nickel-NTA agarose and several chromatographic fractionation steps was devised to purify it to near homogeneity (Fig. 3*C*). Routinely, we could obtain an overall yield of ~2 mg of Rdh54 protein from 150 g of *E. coli* cell paste harvested from 60 liters of culture. Rdh54 protein thus purified from *E. coli* shows a dsDNA-dependent ATPase activity (k_{cat} = 1,500 min^{-1}) similar in potency to that (k_{cat} = 2,200 min^{-1}) of Rdh54 purified from yeast cells, and is also active in DNA supercoiling and in functional interactions with the Rad51 recombinase (see below). Expression of the thioredoxin- and His_6-tagged Rdh54 protein in the haploid *rdh54 K352R* mutant using the *ADH1* promoter in a low copy *CEN* vector complemented the MMS sensitivity of the cells, indicating that the tagged Rdh54 protein is biologically efficacious (data not shown).

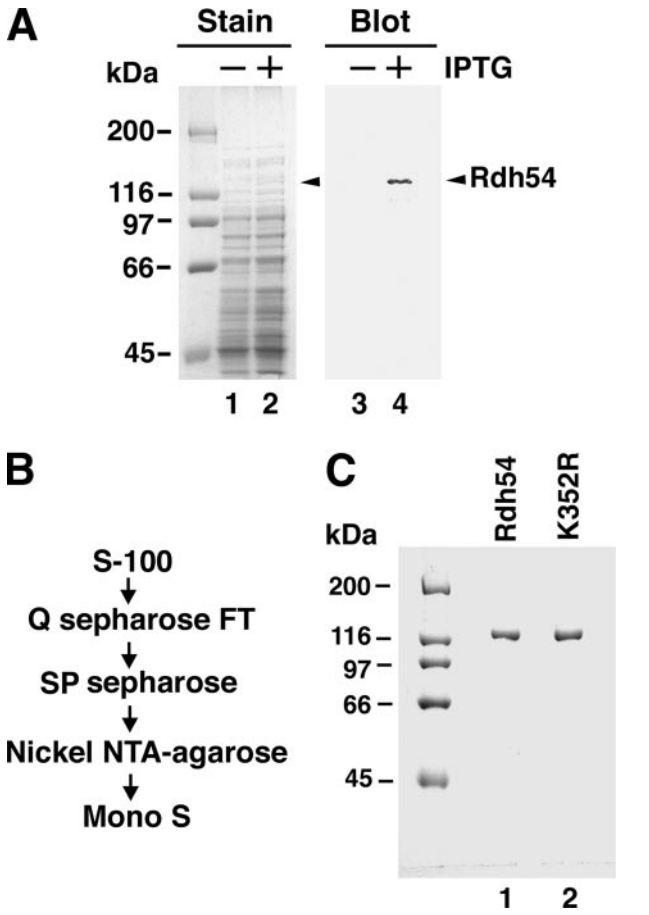

FIGURE 3. **Expression and purification of wild-type Rdh54 and rdh54 K352R proteins.** *A*, extracts from *E. coli* cells harboring pET32a::Rdh54 grown with or without IPTG were analyzed by 7.5% SDS-PAGE and staining with Coomassie Blue (*lanes 1* and *2*) or immunoblotting with anti-histidine antibodies (*lanes 3* and *4*). *B*, scheme for the purification of Rdh54 and rdh54 mutant proteins. *C*, purified Rdh54 (*lane 1*) and rdh54 K352R (*lane 2*), 2 μg each, were resolved by SDS-PAGE and stained with Coomassie Blue.

Following the same overall strategy, we also expressed the rdh54 K352R (erroneously referred to as rdh54 K351R in our previously published work, Ref. 13) mutant protein in *E. coli* and purified it to near homogeneity (Fig. 3*C*) to include in the

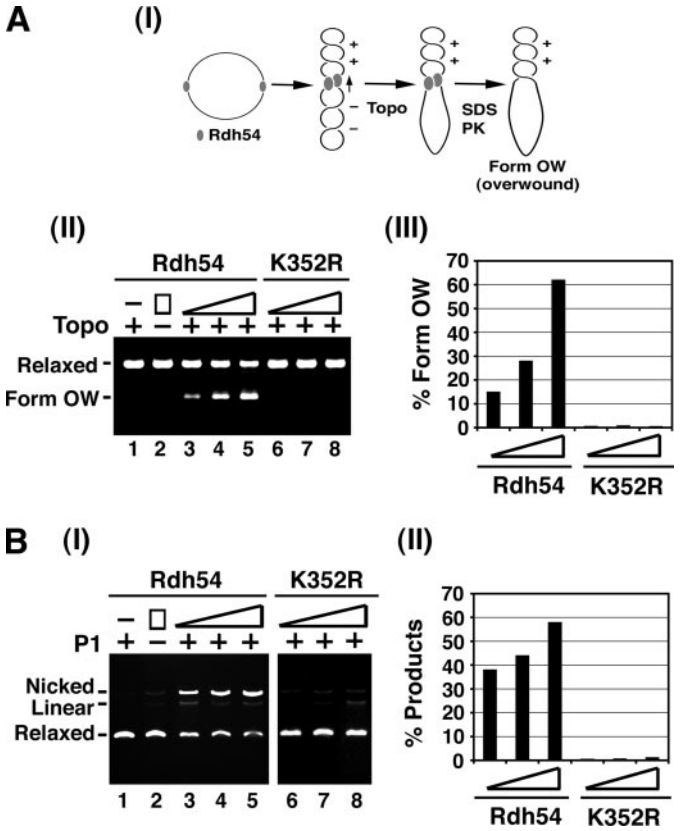

FIGURE 4. **Rdh54 supercoils DNA and promotes DNA strand opening.** *A*, *panel I*, schematic of the topoisomerase I-linked DNA supercoiling assay. Briefly, translocation of Rdh54 on dsDNA generates both positive and negative supercoils, and *E. coli* topoisomerase I removes the negative supercoils, resulting in the formation of a positively supercoiled species, Form OW. *Panel II*, Rdh54 or rdh54 K352R protein (100 nM in *lanes 3* and 6; 150 nM in *lanes 4* and *7*; 200 nM in *lanes 5* and *8*) was incubated with topologically relaxed DNA and topoisomerase (*Topo*). In *lane 1*, the DNA was incubated with topoisomerase but no Rdh54, and in *lane 2*, the DNA was incubated with the highest amount of Rdh54 (200 nM) in the absence of topoisomerase. *Panel III*, results from *panel II* (*lanes 3–8*) are plotted. *B*, *panel I*, Rdh54 or rdh54 K352R protein (300 nM in *lanes 3* and *6*, 400 nM in *lanes 4* and *7*, and 550 nM in *lanes 5* and *8*) was incubated with relaxed DNA and P1 nuclease. In *lane 1*, the DNA was incubated with P1 but no Rdh54, and in *lane 2*, the DNA was incubated with the highest amount of Rdh54 (550 nM) in the absence of P1. *Panel II*, results from *panel I* (*lanes 3–8*) are plotted.

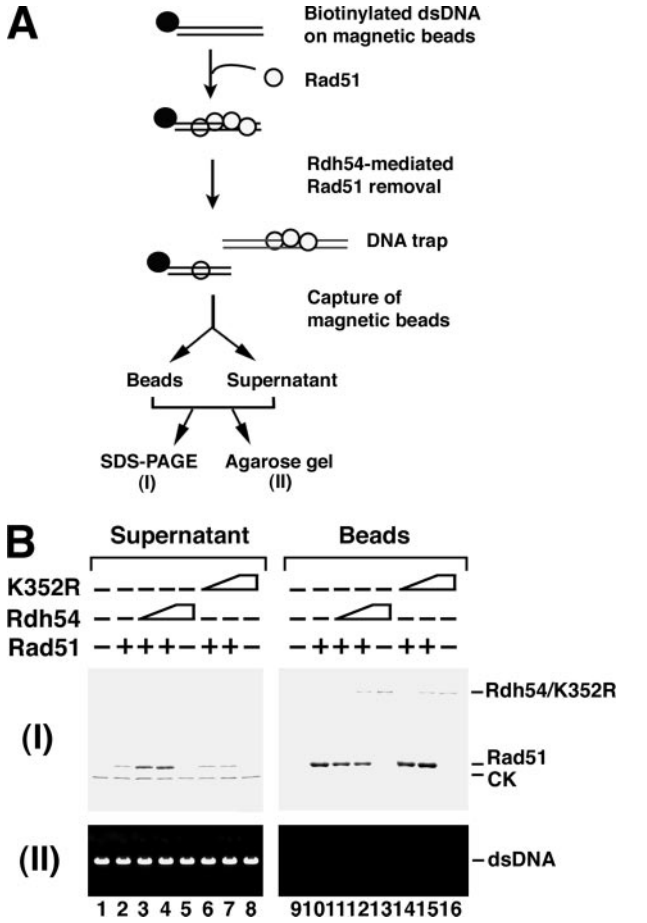

FIGURE 5. **Rdh54 removes Rad51 from dsDNA with a dependence on ATP hydrolysis.** *A*, reaction scheme. Briefly, Rad51-nucleoprotein filaments are assembled on biotinylated dsDNA conjugated to streptavidin magnetic beads. Following the addition of Rdh54, an excess pBluescript dsDNA (DNA trap) is added to trap Rad51 molecules that have been dissociated by Rdh54. The supernatant containing pBluescript dsDNA and trapped Rad51 and the bead-fraction containing Rad51 that remains on the biotinylated dsDNA are subjected to SDS-PAGE or agarose gel electrophoresis to determine their protein or pBluescript dsDNA content, respectively. *B*, supernatant and bead fraction from reactions containing Rad51 and Rdh54 or rdh54 K352R (120 and 240 nM) were analyzed by SDS-PAGE and Coomassie Blue staining (*I*) or agarose gel electrophoresis and ethidium bromide staining (*II*). Control reactions that contained no recombination protein, no Rdh54 or rdh54 K352R, or no Rad51 were also included. *Symbols* in *B*, CK, creatine kinase used in the ATP-regenerating system; dsDNA, the non-biotinylated dsDNA used as Rad51 trap.

biochemical analyses. The rdh54 K352R mutant behaved very much like the wild type protein chromatographically and could be obtained with a similar degree of purity and overall yield. As described below, we have utilized the *E. coli* expression system to obtain various N-terminally truncated variants of Rdh54. The biochemical properties of the full-length and various truncated variants of Rdh54 and their characterization in the context of functional interactions with Rad51 will be presented later. Taken together, the results presented here and later establish *E. coli* as a convenient vehicle for the expression and purification of biologically efficacious Rdh54 protein and mutant variants of this HR factor.

Rdh54 Catalyzes Transient Separation of Strands in Duplex DNA—Through its DNA translocase activity, Rdh54 generates both positive and negative supercoils in the DNA (13) (Fig. 4*A*). Previous studies showed that the related HR protein Rad54 also supercoils DNA in a similar manner (14, 15), and that the negative supercoils produced by this factor render topologically relaxed DNA sensitive to the single-strand specific P1 nuclease because of the transient separation of DNA strands in the DNA duplex (14). In this study, we used the P1 assay to ask whether Rdh54-catalyzed DNA supercoiling also causes DNA strand separation. For this, Rdh54 was incubated with topologically relaxed DNA in the presence of ATP, followed by the addition of P1 and another incubation. The reaction mixtures were deproteinized for analysis in an agarose gel that contained ethidium bromide to resolve the unreacted substrate from DNA digested by the P1 nuclease. As shown in Fig. 4*B*, while, as expected, the relaxed DNA substrate alone was not susceptible to P1 because of the lack of single-stranded nature in the DNA, it became nicked and linearized by P1 in a manner that was proportional to the Rdh54 protein concentration. In concordance with results from our previous work showing the

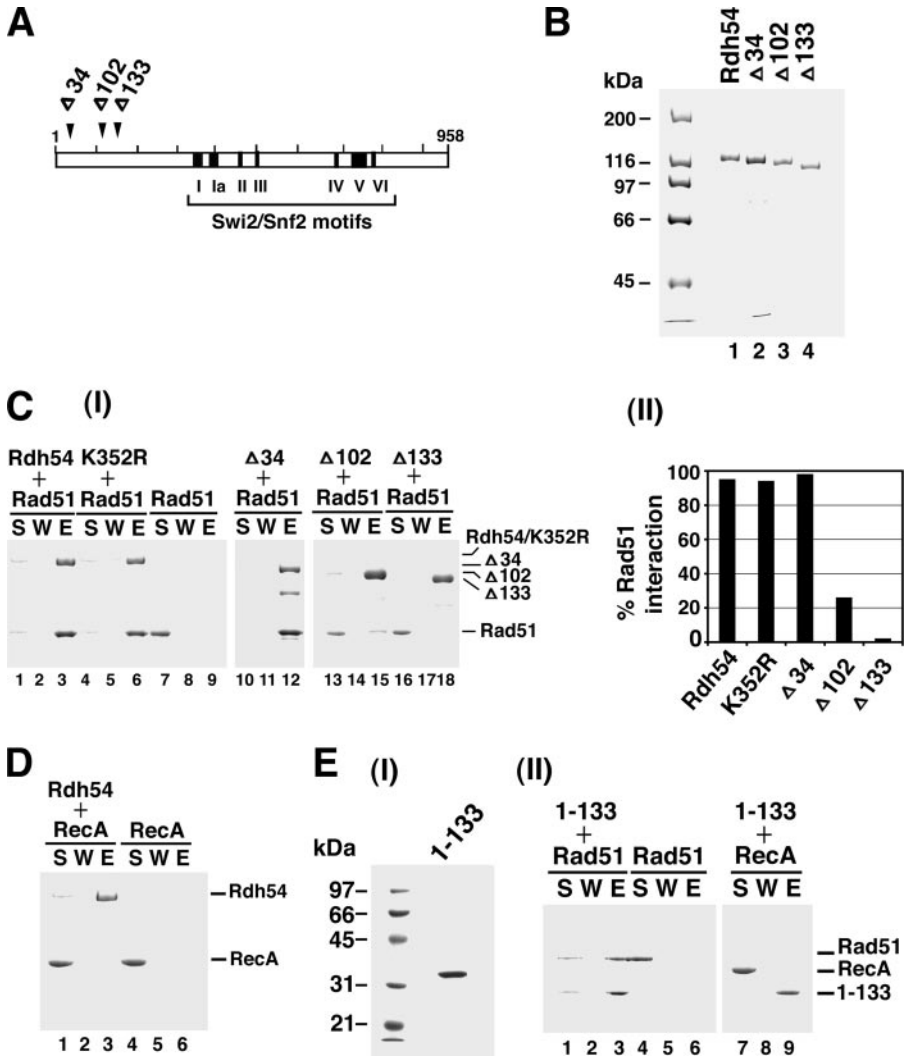

FIGURE 6. **Purification of truncation variants of Rdh54 and examination of their physical interaction with Rad51.** *A*, structure and truncated variants of Rdh54. The *boxed regions* designate the seven helicase-like Swi2/Snf2 motifs in Rdh54. The *arrowheads* mark the N-terminal truncations of Rdh54 that we have constructed. *B*, purified Rdh54 (*lane 1*), rdh54 Δ34 (*lane 2*), rdh54 Δ102 (*lane 3*), and rdh54 Δ133 (*lane 4*), 2 μg each, were resolved by SDS-PAGE and stained with Coomassie Blue. *C*, *panel I*, purified Rdh54 (*lanes 1–3*), rdh54 K352R (*lanes 4–6*), rdh54 Δ34 (*lanes 10–12*), rdh54 Δ102 (*lanes 13–15*), and rdh54 Δ133 (*lanes 16–18*) were incubated with Rad51 and the reaction mixtures subjected to pull-down with Ni^{2+} NTA-agarose to capture protein complexes through the His_6 tag on Rdh54 or the rdh54 mutant variants. The beads were collected by centrifugation, washed with buffer, and then eluted with SDS. The supernatant (*S*), wash (*W*), and SDS-eluate (*E*) were analyzed by 10% SDS-PAGE and staining with Coomassie Blue. Rad51 alone was included as control (*lanes 7–9*). *Panel II*, results from *panel I* (*lanes 3*, *6*, *12*, *15*, and *18*) are plotted. *D*, purified Rdh54 was examined for interaction with RecA (*lanes 1–3*) as described in *C*. RecA alone (*lanes 4–6*) was included as control. *E*, in *panel I*, the purified Rdh54-(1–133) fragment, 2 μg, was subjected to SDS-PAGE and staining with Coomassie Blue. In *panel II*, the purified Rdh54 1–133 fragment was examined for interaction with either Rad51 (*lanes 1–3*) or RecA (*lanes 7–9*) as described in *C*. Rad51 alone (*lanes 4–6*) was included as control.

dependence of the Rdh54-mediated DNA supercoiling reaction on ATP hydrolysis (13), no DNA strand separation was seen upon substitution of the wild-type protein with the rdh54 K352R variant that has no ATPase activity. Taken together, the results show that the negative supercoiling generated by the Rdh54 DNA translocase activity leads to unwinding of the DNA helix.

Rdh54 Removes Rad51 from DNA with a Dependence on ATP Hydrolysis—Rad54, also a Swi2/Snf2-like HR factor, uses its DNA translocase activity to dislodge Rad51 from dsDNA (33). Likewise, the *S. cerevisiae* DNA helicase Srs2 removes Rad51 protein encountered in its path of translocation on ssDNA (34, 35). Because Rdh54 protein possesses a dsDNA translocase activity, we wished to examine whether it too can remove Rad51 from dsDNA. We first devised an assay that would allow us to monitor the dissociation of the Rad51-dsDNA nucleoprotein filament and quantify the efficiency of the reaction. In this assay system (Fig. 5*A*), Rad51 is incubated with streptavidin magnetic beads that contain a 600-bp biotinylated dsDNA fragment to allow for Rad51-nucleoprotein filament assembly on the DNA, followed by the addition of Rdh54. After a brief incubation, an excess of linear, non-biotinylated dsDNA is incorporated to trap the Rad51 molecules that have been displaced from the magnetic bead-linked dsDNA by Rdh54. The supernatant containing Rad51 trapped on non-biotinylated DNA and the SDS eluate of the magnetic beads harboring Rad51 associated with the biotinylated DNA are subjected to SDS-PAGE to quantify their Rad51 content. These fractions are also analyzed by agarose gel electrophoresis and ethidium bromide staining to verify that there is no unwanted aggregation of the non-biotinylated DNA with the magnetic bead-bound material.

As shown in Fig. 5*B*, there was a Rdh54 concentration-dependent transfer of Rad51 protein from the magnetic bead-bound DNA to the non-biotinylated dsDNA trap, indicative of dissociation of the Rad51-dsDNA nucleoprotein filament by Rdh54. The Rdh54-mediated removal of Rad51 from dsDNA requires ATP hydrolysis by the former, as no such transfer occurred when we substituted Rdh54 with the rdh54 K352R mutant variant that is proficient in Rad51 interaction but defective in ATP hydrolysis (13).

Construction, Expression, and Purification of N-terminally Truncated rdh54 Mutants—In Rdh54, the core of the catalytic domain harboring the seven conserved Swi2/Snf2 motifs that are concerned with DNA binding and ATP hydrolysis (36–39) is located a good distance away from the N-terminal portion (Fig. 6*A*). It seems likely that the N-terminal portion of Rdh54 confers the ability to interact with other HR factors. For testing the role of this N-terminal domain in complex formation and

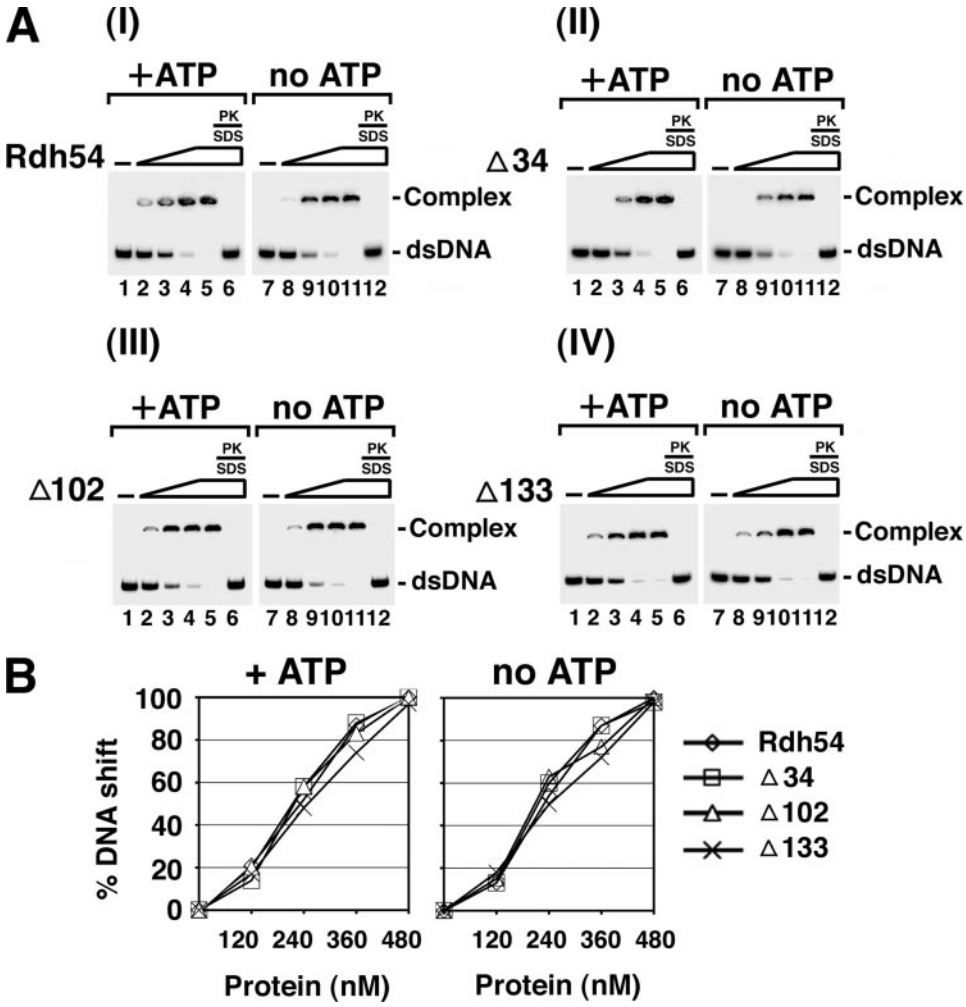

FIGURE 7. **DNA binding by Rdh54 and its truncation variants.** *A*, increasing amounts (120 nM in *lanes 2* and *8*, 240 nM in *lanes 3* and *9*, 360 nM in *lanes 4* and *10*, and 480 nM in *lanes 5* and *11*) of Rdh54 (*panel I*) and the Δ34 (*panel II*), Δ102 (*panel III*), and Δ133 (*panel IV*) truncation variants were incubated with ^{32}P-labeled DNA with or without ATP, as indicated. In *lanes 6* and *12*, a reaction containing the highest amount of Rdh54 or rdh54 mutant (480 nM) was deproteinized by SDS and proteinase K (*PK*). The reaction mixtures were subjected to polyacrylamide gel electrophoresis and phosphorimaging analysis. *B*, results in *A* are plotted.

functional interactions with Rad51, we constructed truncation mutants of Rdh54, deleting 34, 102, or 133 amino acid residues from the N terminus. The rdh54 Δ102 and rdh54 Δ133 truncation mutants can be expressed in *E. coli*, are soluble, and can be purified to near homogeneity (Fig. 6*B*) using the same chromatographic procedure (Fig. 3*B*) that we have developed for the full-length protein. The overall yield of the two N-terminally truncated rdh54 mutants is similar to that of the full-length protein. The rdh54 Δ34 mutant protein can also be expressed in *E. coli* and is soluble, but it proves to be more susceptible to intracellular proteolysis. The major proteolytic product of rdh54 Δ34 has a size of 70 kDa. This proteolytic product co-purified with rdh54 Δ34 and represented between 10 and 30% of the final purified preparation. The overall yield of rdh54 Δ34 protein is similar to that of full-length Rdh54.

The Rad51 Binding Domain Is Located within the N Terminus of Rdh54—To examine whether the N-terminally truncated rdh54 proteins are capable of Rad51 interaction, we incubated the full-length and the N-terminally truncated variants of Rdh54 with Rad51 and then captured protein complexes on nickel-NTA agarose beads through the His6 affinity tag on the Rdh54 species. The nickel-NTA agarose beads were treated with SDS to elute bound proteins, followed by SDS-PAGE. As reported before (13) and shown here in Fig. 6*C*, full-length Rdh54 interacted with Rad51 avidly. The rdh54 Δ34 protein bound about the same amount of Rad51 as full-length Rdh54, whereas the rdh54 Δ102 truncation mutant had a much weaker affinity for Rad51, and the rdh54 Δ133 protein was completely defective in this regard. Neither full-length Rdh54 (Fig. 6*D*) nor any of the truncation rdh54 mutants (data not shown) bound *E. coli* RecA protein.

The above results indicated that the N-terminal 133 residues of Rdh54 are necessary for Rad51 binding. We wished to ascertain whether this N-terminal region of Rdh54 is sufficient for Rad51 interaction. To accomplish this goal, we expressed and purified to near homogeneity the Rdh54 fragment that encompasses the N-terminal 133 residues as a His_6-tagged polypeptide (Fig. 6*E*, *panel I*). Importantly, the Rdh54-(1–133) fragment exhibited the same high affinity for Rad51 as full-length Rdh54 but, as expected, did not bind the *E. coli* RecA protein (Fig. 6*E*, *panel II*).

Biochemical Attributes of Rdh54 and N-terminally Truncated Variants—We examined the purified rdh54 Δ34, Δ102, and Δ133 proteins for the biochemical attributes of Rdh54, *i.e.* DNA binding, DNA-dependent ATP hydrolysis, DNA supercoiling, and DNA strand opening. As shown in Figs. 7 and 8, the three N-terminally truncated proteins are just as proficient as full-length Rdh54 in all these aspects, indicating that the truncations have no undesirable effect on the basic biochemical functions of Rdh54. We also tested the rdh54 K352R protein for DNA binding and found that it is just as proficient as the full-length and truncated forms in this regard (data not shown).

Rdh54·Rad51 Complex Formation Is Critical for Functional Interactions—Full-length Rdh54 purified from *E. coli* can greatly enhance the ability of Rad51 to make D-loop (Fig. 9), just as what we previously documented for Rdh54 purified from yeast (13). Even though the rdh54 Δ34 protein retains the ability to bind Rad51 (Fig. 6*C*), it is, reproducibly, less effective than full-length Rdh54 in the D-loop reaction (Fig. 9). Importantly, the rdh54 Δ102 mutant, which is significantly impaired for

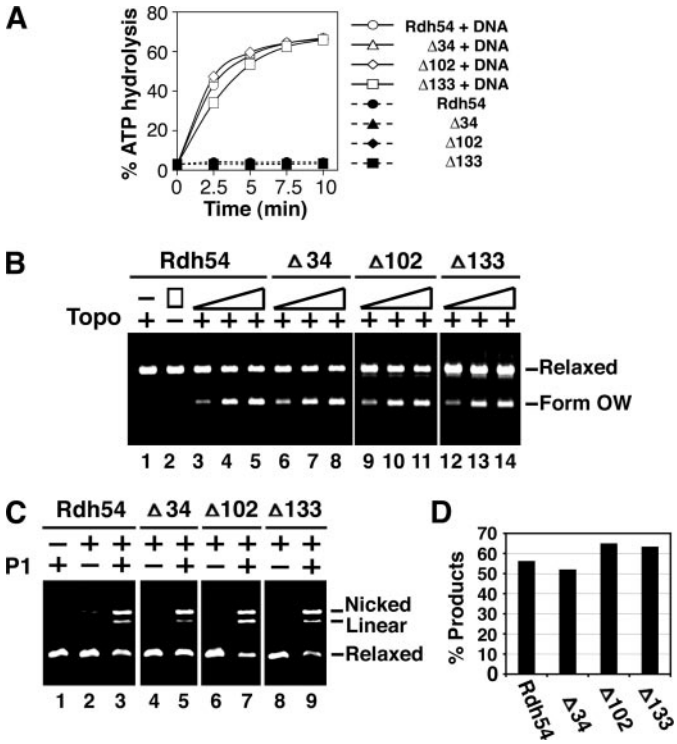

FIGURE 8. **ATP hydrolysis, DNA topology modification, and DNA strand opening by Rdh54 and its truncation variants.** *A*, examine ATP hydrolysis, Rdh54 and the Δ34, Δ102, and Δ133 truncation variants, 150 nM each, were incubated with [γ-^{32}P]ATP and with or without DNA for the indicated times. Analysis was by thin layer chromatography. *B*, to assess DNA supercoiling activity, increasing amounts (100 nM in *lanes 3*, *6*, *9*, and *12*; 150 nM in *lanes 4*, *7*, *10*, and *13*; 200 nM in *lanes 5*, *8*, *11*, and *14*) of Rdh54 and the Δ34, Δ102, and Δ133 truncation variants were incubated with topologically relaxed DNA and *E. coli* topoisomerase I and then subjected to agarose gel electrophoresis and ethidium bromide staining, as described in the Fig. 4 legend. In *lane 1*, the DNA was incubated with topoisomerase but no Rdh54, and in *lane 2*, the DNA was incubated with Rdh54 but no topoisomerase. *C*, to examine DNA strand opening, Rdh54 and the Δ34, Δ102, and Δ133 truncation variants (550 nM each) were incubated with relaxed DNA and with or without P1 nuclease, as indicated. In *lane 1*, the DNA was incubated with P1 but no Rdh54. *D*, nicked and linear DNA forms of DNA from the experiment in *C* (*lanes 3*, *5*, *7*, and *9*) are quantified and plotted.

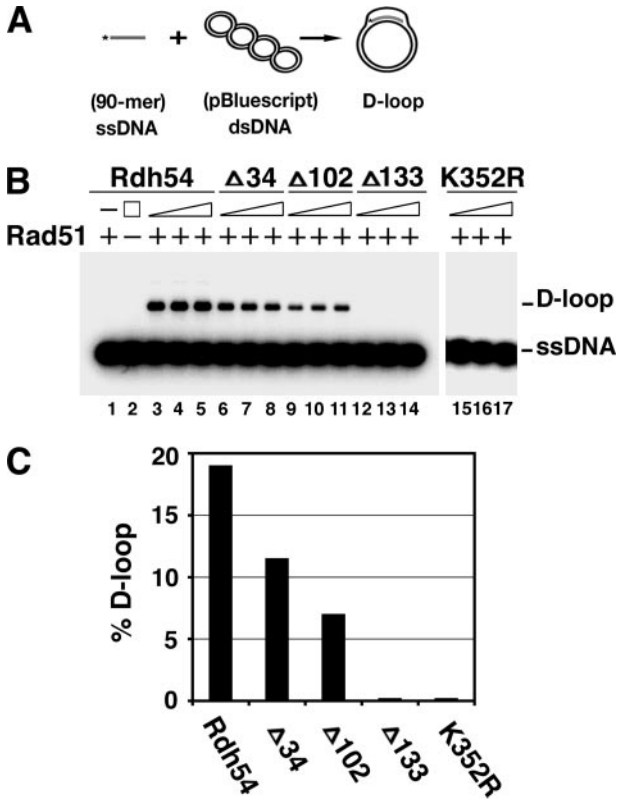

FIGURE 9. **Dependence of the D-loop reaction on the N-terminal Rad51 binding domain of Rdh54.** *A*, reaction schematic. Pairing between a radiolabeled 90-mer single strand DNA (*ssDNA*) and homologous replicative form I pBluescript DNA yields a D-loop. *B*, D-loop reactions containing Rad51 and increasing amounts of Rdh54, rdh54 K352R, and the rdh54 Δ34, Δ102, and Δ133 truncation mutants (300 nM in *lanes 3*, *6*, *9*, *12*, and *15*; 400 nM in *lanes 4*, *7*, *10*, *13*, and *16*; 500 nM in *lanes 5*, *8*, *11*, *14*, and *17*). The reaction mixtures were deproteinized and then subjected to agarose gel electrophoresis and phosphorimaging analysis. In *lane 1*, the DNA substrates were incubated with Rad51 alone, and in *lane 2*, the DNA substrates were incubated in buffer with Rdh54 alone. *C*, results from the experiment in *B* (*lanes 5*, *8*, *11*, *14*, and *17*) are plotted.

Rad51 interaction (Fig. 6*C*), is much less capable of promoting the D-loop reaction, while the rdh54 Δ133 mutant, which is devoid of Rad51 binding ability (Fig. 6*C*), is completely defective in this regard (Fig. 9). These results support the premise that Rdh54·Rad51 complex formation is a prerequisite for functional cooperation of these two HR factors in the D-loop reaction.

In addition to examining D-loop formation, we also asked whether the Rdh54 ATPase, DNA supercoiling, and DNA strand opening activities can be enhanced by Rad51. Reproducibly, a significantly higher level of ATP hydrolysis was seen upon mixing Rad51 with full-length Rdh54 (Fig. 10*A*). The enhancement is caused by up-regulation of the Rdh54 ATPase activity by Rad51, as a very similar result was obtained when we substituted Rad51 with the rad51 K191R protein (30) that lacks ATPase activity (Fig. 10*A*). In general agreement with the results from examining D-loop formation, ATP hydrolysis by rdh54 Δ34 is stimulated to a lesser degree by Rad51 (Fig. 10*A*), and that the ATPase activity of rdh54 Δ102 (data not shown) or rdh54 Δ133 (Fig. 10*A*) is refractory to Rad51. Reproducibly, Rad51 stimulates the DNA supercoiling activity of full-length Rdh54 slightly (Fig. 10*B*) but exerts no perceptible effect on the reaction mediated by the rdh54 truncation mutants, *i.e.* Δ34, Δ102, and Δ133 (data not shown). We consistently saw a 2-fold enhancement of the DNA strand opening activity of full-length Rdh54 by Rad51 (Fig. 10*C*), but the reaction mediated by the rdh54 truncation mutants is not influenced by Rad51 (data not shown).

Partial Dependence of Rdh54-mediated Rad51 Removal from DNA on Protein Complex Formation—Results presented earlier show that Rdh54 efficiently removes Rad51 protein from dsDNA in a reaction that is linked to ATP hydrolysis by the former (Fig. 5*B*). We used the same assay system to query whether the three truncated variants: Δ34, Δ102, and Δ133 are capable of removing Rad51 from DNA. To do this, increasing amounts of the truncated rdh54 mutants were incubated with Rad51 filaments assembled on magnetic bead-bound DNA in the presence of a non-biotinylated dsDNA used as Rad51 trap (see Fig. 5*A* for schematic). The analysis revealed Rad51 dissociation from the bead-bound DNA by rdh54 Δ34 and rdh54 Δ102 with an efficiency very similar to that seen with full-length Rdh54 (Fig. 11). In contrast, the rdh54 Δ133 is partially

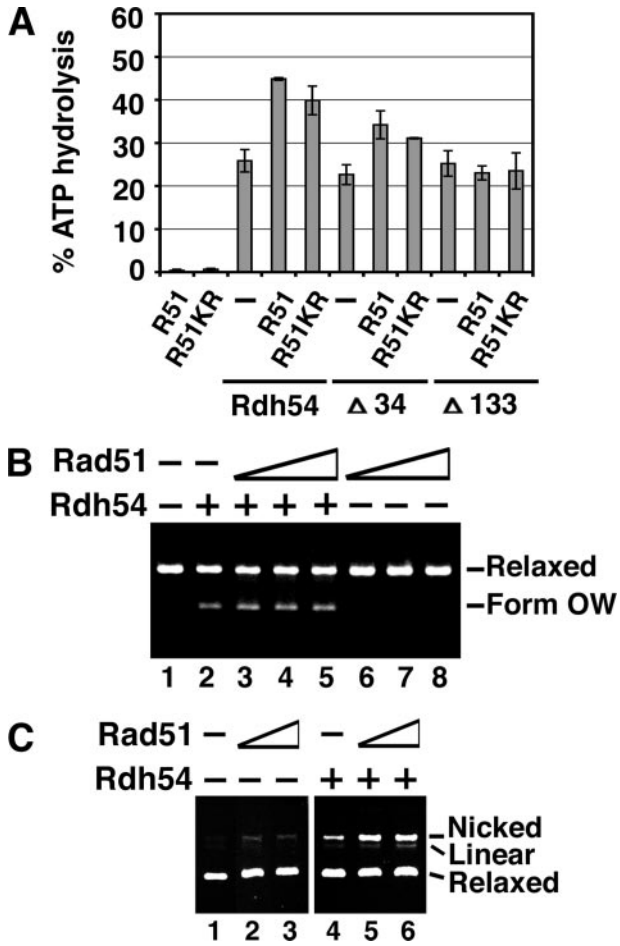

FIGURE 10. **Functional interaction of Rdh54 and rdh54 truncation variants with Rad51 in ATP hydrolysis and DNA topology modification.** *A*, to examine the effect of Rad51 or rad51 K191R on ATPase hydrolysis by Rdh54 and rdh54 truncation (Δ34 and Δ133) mutants, these proteins (40 nM each) were incubated with [γ-^{32}P]ATP, dsDNA, and with or without Rad51 (R51) or rad51 K191R (R51KR), 500 nM each, in reaction buffer for 10 min. Rad51 and rad51 K191R were also incubated without Rdh54. Quantification was by thin layer chromatography. *B*, to examine the effect of Rad51 on DNA topology modification by Rdh54, increasing amounts of Rad51 (0.5, 0.75, and 1 μM in *lanes 3–5*, respectively) was incubated with Rdh54 (80 nM), topologically relaxed DNA, and *E. coli* topoisomerase I. For controls, the DNA substrate was incubated with topoisomerase alone (*lane 1*) and with the same amounts of Rad51 (0.5, 0.75, and 1 μM in *lanes 8–11*, respectively) and topoisomerase but without Rdh54. *C*, to examine the effect of Rad51 on DNA strand opening by Rdh54, Rad51 (0.75 and 1 μM in *lanes 4* and *5*, respectively) was incubated with Rdh54 (150 nM), topologically relaxed DNA and P1 nuclease. For controls, the DNA substrate was incubated with P1 alone (*lane 1*) and with the same amounts of Rad51 (0.75 and 1 μM in *lanes 2* and *3*, respectively) and P1 but without Rdh54.

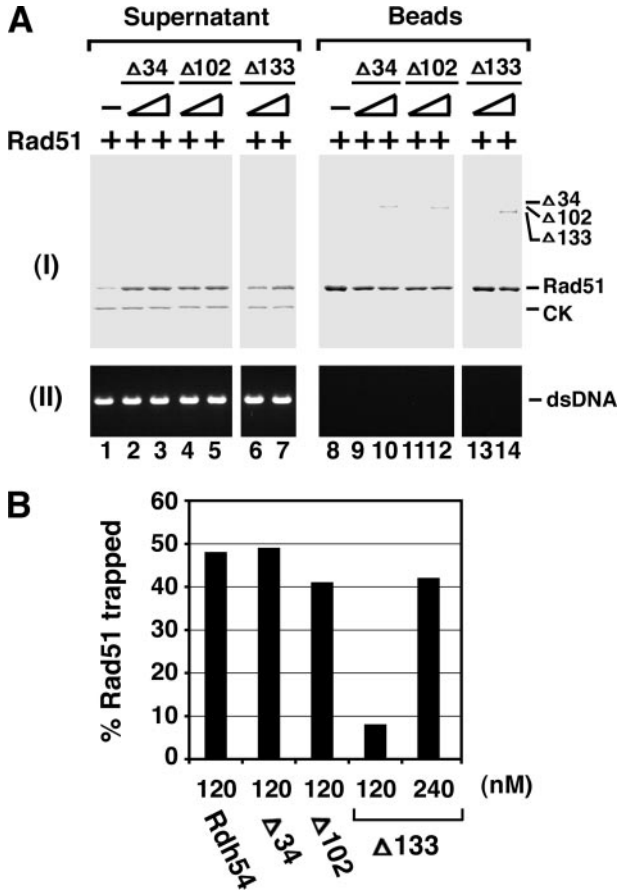

FIGURE 11. **Role of the Rdh54 N-terminal Rad51 binding domain in Rad51 removal from DNA.** *A*, to examine Rad51 removal from DNA, Rad51-nucleoprotein filaments assembled on magnetic bead-bound dsDNA were incubated with the rdh54 Δ34, Δ102, and Δ133 truncation mutants (120 or 240 nM) and the dsDNA trap. Analysis was by either SDS-PAGE (I) or agarose gel electrophoresis (II). The control reaction in *lane 1* contained Rad51 only. See Fig. 5 legend for experimental details. Symbols: *CK*, creatine kinase in the ATP-regenerating system used; *dsDNA*, the non-biotinylated dsDNA used as Rad51 trap. *B*, results from Fig. 5*B* (*lane 3*) and *A* (*lanes 2*, *4*, *6*, and *7*) are plotted.

impaired for this activity, seen as a reduced efficiency of Rad51 removal at lower rdh54 protein concentrations (Fig. 11). Thus, Rad51 removal by Rdh54 occurs in the absence of the N-terminal Rad51 interaction domain in the latter, albeit with reduced efficiency.

DISCUSSION

Genetic studies conducted in several laboratories have shown a role of the *RDH54* gene in mitotic and meiotic HR, specifically in those events that involve homologous chromosomes, *i.e.* interhomologue recombination (9, 10). Despite the importance of *RDH54* in inter-homologue recombination, there is only limited information concerning the biochemical properties of the Rdh54 protein or its functional interactions with Rad51. Biochemical studies involving Rdh54 and Dmc1 have not yet been carried out. The lack of rapid advance in the dissection of the HR function of Rdh54 stems in large part from the lack of a convenient Rdh54 protein expression vehicle. Herein, we have described an *E. coli* expression system that, with the aid of a straightforward purification procedure, yields milligram quantities of nearly homogeneous protein. Using the purified material, we have shown a DNA strand opening activity in Rdh54. This DNA strand opening activity requires ATP hydrolysis by Rdh54 and is very likely caused by the negative supercoiling generated as a result of translocation of Rdh54 on dsDNA (13). In this regard, Rdh54 resembles Rad54 and other Swi2/Snf2-like factors that have been examined to date (14–16, 18). DNA supercoiling and associated strand opening mediated by Rdh54 (13) (this work) are expected to enhance the likelihood of DNA joint formation by the Rad51 presynaptic filament during HR, as previously suggested for Rad54 (14, 15, 40).

We have employed the same *E. coli* expression system and purification protocol to prepare nearly homogenous rdh54 mutant proteins as well, including the ATPase defective rdh54

K352R and several N-terminally truncated variants. Deletion of as many as 133 residues from the N terminus of Rdh54 has no negative impact on the basic biochemical attributes (*i.e.* DNA binding, ATPase, DNA supercoiling, and DNA strand opening activities) of the protein. With the rdh54 truncation mutants and a polypeptide comprising the first 133 residues of Rdh54, we have demonstrated that the Rad51 binding domain resides within the N terminus of Rdh54, and that functional interactions of Rdh54 and Rad51 in the D-loop reaction in ATP hydrolysis, DNA supercoiling, and DNA strand opening by Rdh54 require the N terminus of Rdh54. This finding and the fact that Rdh54 neither binds nor functionally synergizes with the *E. coli* RecA protein (13) (this work) are consistent with the premise that specific complex formation between Rdh54 and Rad51 is a prerequisite for functional interactions between these two *S. cerevisiae* HR factors. Previous studies have shown that complex formation of Rad54 with Rad51 is required for functional synergy between these two recombination factors (14, 33, 38, 39, 41–44), and Rdh54 (13) (this work) resembles Rad54 in this regard.

Rad54 dislodges Rad51 from dsDNA in a reaction that requires ATP hydrolysis by Rad54 (33). This activity of Rad54 is thought to mediate the removal of Rad51 from the nascent D-loop structure so as to expose the primer end in the D-loop for the initiation of repair DNA synthesis (33), which is a critical step in the HR reaction (1, 8). Alternatively, or in addition, Rad54 may release Rad51 from bulk chromatin and from heteroduplex DNA joints made during HR reactions so as to maximize the free pool of Rad51 to be utilized for HR and DNA repair reactions. In this study, we have presented data to show an ability of Rdh54 to dissociate Rad51 from dsDNA with a strict dependence on the ATPase activity of Rdh54. It seems possible that this Rdh54 activity likewise promotes the intracellular recycling of Rad51 and helps initiate DNA synthesis during interhomologoue recombination and DNA repair reactions. Our results demonstrate that the Rdh54-mediated removal of Rad51 from DNA is only partially reliant on the Rad51 binding domain in Rdh54. Even though Rad51 removal in the *in vitro* setting is not absolutely contingent upon Rdh54 possessing the ability to bind Rad51, within cells, complex formation with Rad51 may well be necessary for efficient targeting of Rdh54 to chromosome locales where Rad51 is bound. As noted earlier, complex formation of Rad54 with Rad51 appears to be indispensable for functional synergy between them (14, 33, 38, 39, 41–44). It will be important to test whether variants of Rad54 that lack the ability to interact with Rad51 (38, 39) are capable of clearing Rad51 from dsDNA.

Our biochemical studies (13) (this work) have shown an indispensable role of the Rdh54 ATPase activity in DNA supercoiling, DNA strand opening, removal of Rad51 from DNA, and the D-loop reaction. Accordingly, genetic studies involving the *rdh54 K352R* allele as presented here have revealed a biological requirement of the Rdh54 ATPase function in recombination and DNA repair in mitotic and meiotic cells. Interestingly, in both the haploid and diploid states, the *rdh54 K352* mutation causes a degree of MMS sensitivity significantly higher than that engendered by deleting *RDH54*. This finding argues that a non-productive complex of rdh54 K352R mutant protein and its partner protein(s) is deleterious to the repair of DNA damage induced by MMS.

Several members of the Swi2/Snf2 protein family, including Rad54 (45), possess a chromatin remodeling activity (46). In the case of Rad54, chromatin remodeling is stimulated by a specific interaction with Rad51 (42, 43, 47). With the Rdh54 expression and purification systems that we have devised, it will be possible to address whether Rdh54 also has a chromatin remodeling activity and if this activity is enhanced by Rad51.

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