

Biochemical Analysis of the DNA Unwinding and Strand Annealing Activities Catalyzed by Human RECQ1*[§]

Received for publication, January 7, 2005, and in revised form, May 16, 2005
Published, JBC Papers in Press, May 16, 2005, DOI 10.1074/jbc.M500264200

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RecQ helicases play an important role in preserving genomic integrity, and their cellular roles in DNA repair, recombination, and replication have been of considerable interest. Of the five human RecQ helicases identified, three are associated with genetic disorders characterized by an elevated incidence of cancer or premature aging: Werner syndrome, Bloom syndrome, and Rothmund-Thomson syndrome. Although the biochemical properties and protein interactions of the WRN and BLM helicases defective in Werner syndrome and Bloom syndrome, respectively, have been extensively investigated, less information is available concerning the functions of the other human RecQ helicases. We have focused our attention on human RECQ1, a DNA helicase whose cellular functions remain largely uncharacterized. In this work, we have characterized the DNA substrate specificity and optimal cofactor requirements for efficient RECQ1-catalyzed DNA unwinding and determined that RECQ1 has certain properties that are distinct from those of other RecQ helicases. RECQ1 stably bound to a variety of DNA structures, enabling it to unwind a diverse set of DNA substrates. In addition to its DNA binding and helicase activities, RECQ1 catalyzed efficient strand annealing between complementary single-stranded DNA molecules. The ability of RECQ1 to promote strand annealing was modulated by ATP binding, which induced a conformational change in the protein. The enzymatic properties of the RECQ1 helicase and strand annealing activities are discussed in the context of proposed cellular DNA metabolic pathways that are important in the maintenance of genomic stability.

Cellular processes such as DNA replication, recombination, and repair often involve steps that require unwinding of double-stranded DNA (dsDNA)¹ to form transient single-stranded

DNA (ssDNA) intermediates. Helicases are a class of enzymes that unwind DNA duplexes with a distinct directional polarity, either 3' to 5' or 5' to 3' with respect to the strand on which the helicase is presumed to translocate, deriving energy from the hydrolysis of ATP (1, 2). An essential "caretaker" role of helicases in DNA metabolic processes is suggested by a number of genetic disorders that have been linked to defects in DNA helicases (3–5).

The RecQ family of DNA helicases, named after the *Escherichia coli* RecQ DNA helicase, is highly conserved from bacteria to humans (4). RecQ helicases share a centrally located helicase domain of ~450 residues containing the seven conserved helicase motifs and an additional conserved region of ~80 amino acids located C-terminal to the helicase domain designated RecQ-Ct (4, 6). The conserved helicase domain couples nucleotide hydrolysis to DNA unwinding, whereas the RecQ-Ct domain is suggested to be involved in protein interaction (7) and DNA binding (8). In all organisms, RecQ helicases play critical roles in maintaining genomic stability as evidenced by the hyper-recombination phenotype observed in *recQ* mutants (for review, see Ref. 9).

There are five human helicases with the defined region of homology in the conserved RecQ helicase motifs: 1) WRN, defective in Werner syndrome; 2) BLM, defective in Bloom syndrome; 3) RTS, defective in Rothmund-Thomson syndrome; 4) RECQ1; and 5) RECQ5 (3). Werner syndrome, Bloom syndrome, and Rothmund-Thomson syndrome are all characterized as genomic instability disorders with an elevated cancer incidence and/or premature aging. Human diseases have not yet been genetically linked to mutations in RECQ1 or RECQ5; however, these proteins are likely to have important roles in nucleic acid metabolism based on the biological significance of other RecQ helicases.

The biological importance of RECQ1 cannot be clearly evaluated at present because there is little information on the phenotypes of human *RECQ1* mutant cells. Recent genetic complementation studies using the chick DT40 cell-based system indicate that *RECQ1*^{-/-} cells do not display a growth deficiency, sensitivity to DNA damage, or elevated sister chromatid exchange; however, *RECQ1*^{-/-} *BLM*^{-/-} cells grow more slowly compared with *BLM*^{-/-} cells due to an increased population of dead cells, indicating that RECQ1 is involved in cell viability under the BLM-impaired condition (10). In addition, the double mutant *RECQ1*^{-/-} *BLM*^{-/-} cells exhibit elevated mitomycin C-induced sister chromatid exchange compared with the *BLM*^{-/-} cells, suggesting that RECQ1 may have some redundant functions with the BLM helicase (10).

Recent studies of BLM function in *Drosophila* suggests a role of the helicase in double-strand break repair by a homologous recombination pathway known as synthesis-dependent strand

* The work was supported in part by a grant from the Human Frontier Science Program, by an Fondogli Investimenti della Ricerca di Base (FIRB) grant from the Ministero dell'Istruzione dell'Università e della Ricerca, and by Grant 02.00648.ST97 from the Consiglio Nazionale delle Ricerche (Rome). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–3.

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¹ The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SDSA, synthesis-dependent strand annealing; RPA, replication protein A; HJ, Holliday junction; ATP_γS, adenosine 5'-O-(thiotriphosphate).

annealing (SDSA) (11, 12). This pathway exploits the cellular recombination machinery to rejoin double-strand breaks through a series of strand invasion, DNA synthesis, and re-annealing steps. Although the precise details of BLM function in SDSA are not well understood, the ability of BLM to unwind D-loop structures (13), a key intermediate of the SDSA pathway, is likely to be relevant. Consistent with this, the *Drosophila* genetic data support a model in which BLM acts downstream of strand invasion (12). Understanding the molecular functions of RECQ1 might provide some insight as to how the enzyme has at least partially redundant or synergistic functions with BLM in a pathway such as SDSA.

Recent biophysical and biochemical characterization of the purified recombinant RECQ1 helicase provided evidence that RECQ1 forms dimers in solution (14) and is able to unwind short 3'-ssDNA tailed duplex substrates (14, 15). Considerably longer substrates have been shown to be unwound in the presence of the human ssDNA-binding protein replication protein A (RPA) (14, 15). In this study, we have investigated the unwinding of various DNA structures by RECQ1 helicase. In the course of this work, we determined that RECQ1 has the ability to form stable protein complexes with either ssDNA or dsDNA, suggesting that RECQ1 may utilize its physical interaction with DNA for functions other than duplex DNA unwinding. A novel strand annealing activity of human RECQ5 β helicase was reported recently (16). Because RECQ1 is able to efficiently bind ssDNA, we became interested in the possibility that RECQ1 might utilize its ssDNA binding activity to bridge the complementary ssDNA molecules and to promote strand annealing. The results presented here provide evidence that RECQ1 can efficiently catalyze DNA unwinding of model recombination/repair DNA intermediates or promote strand annealing of complementary ssDNA molecules and that these processes are modulated by nucleotide availability, which alters RECQ1 protein conformation. The dual catalytic functions of RECQ1 are likely to be important in a cellular DNA metabolic pathway important for maintenance of genomic stability.

MATERIALS AND METHODS

Proteins—Recombinant His-tagged RECQ1 protein was overexpressed using a baculovirus/Sf9 insect system and purified to near homogeneity as described previously (14). A baculovirus encoding mutant recombinant His-tagged RECQ1-K119A protein was constructed as described (17), and the RECQ1-K119A protein was purified using the same procedure as described for wild-type recombinant RECQ1 protein. Purified recombinant human RPA was kindly provided by Dr. Mark Kenny (Albert Einstein Medical Center, Bronx, NY). Purified recombinant RuvA protein was kindly provided by Dr. Michael Cox (University of Wisconsin, Madison, WI). *E. coli* ssDNA-binding protein was purchased from Promega.

DNA Substrates—PAGE-purified oligonucleotides used for preparation of DNA substrates were purchased from Midland Certified Reagent Co. 5'-³²P-labeled duplex DNA substrates were prepared as described previously (18). Synthetic Holliday junction HJ(X12) was made by annealing four 50-mer oligonucleotides (X12-1, X12-2, X12-3, X12-4) as described previously (19). D-loop substrates were prepared as described previously (13).

DNA Helicase Assays—Helicase reaction mixtures (20 μ l) contained 20 mM Tris-HCl (pH 7.5), 10 mM KCl, 8 mM dithiothreitol, 5 mM MgCl₂, 5 mM ATP (unless indicated otherwise), 10% glycerol, 80 μ g/ml bovine serum albumin, 0.5 nM DNA substrate, and the indicated RECQ1 concentrations. Reactions were initiated by the addition of RECQ1 unless noted otherwise, and the reaction mixtures were incubated for the indicated times at 37 °C. In kinetic experiments, 200- μ l reactions containing forked duplex substrate (0.8 or 3.2 nM, as specified) and either 10 or 20 nM RECQ1 were initiated with 5 mM ATP, and 20- μ l aliquots were removed at 0–180 s. Unless indicated otherwise, helicase reactions were terminated by the addition of 20 μ l of stop buffer (35 mM EDTA, 0.6% SDS, 25% glycerol, 0.04% bromophenol blue, and 0.04% xylene cyanol) with a 10-fold molar excess of unlabeled competitor oligonucleotide to prevent re-annealing of the unwound ssDNA products. Unlabeled competitor oligonucleotide was not included in the

quench for helicase reactions with D-loop or HJ substrates. Reaction products were subsequently incubated with 0.1 mg/ml proteinase K for 15 min at 37 °C, and the products of standard duplex and D-loop substrates were resolved on non-denaturing 12% polyacrylamide gels. Products of the HJ branch migration reactions were resolved on native 10% polyacrylamide gels. Radiolabeled DNA species on polyacrylamide gels were visualized with a PhosphorImager and quantitated using ImageQuant software (Amersham Biosciences). The percent of helicase substrate unwound was calculated by the following formula: percent unwinding = $100 \times (P/(S + P))$, where P is the product and S is the residual substrate. The values of P and S were corrected after subtracting background values in controls with no enzyme and heat-denatured substrate, respectively. Helicase data represent the mean of at least three independent experiments, with mean \pm S.D. indicated by error bars.

DNA Binding Assays—Reaction mixtures (20 μ l) contained the indicated amounts of RECQ1 and 0.5 nM ³²P-end-labeled DNA substrates in DNA binding buffer (20 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 20 mM NaCl, 0.1 mg/ml bovine serum albumin, and 1 mM dithiothreitol) containing 1 mM ATP γ S or no nucleotide. The reactions were incubated at 24 °C for 15 min after the addition of RECQ1. After incubation, 3 μ l of loading dye (74% glycerol, 0.01% xylene cyanol, and bromophenol blue) was added to each reaction, and samples were loaded onto native 5% polyacrylamide gels (19:1 cross-linking ratio) and electrophoresed at 200 V for 2.5 h at 4 °C using 1 \times Tris borate/EDTA as the running buffer. The resolved radiolabeled species were visualized with a PhosphorImager and analyzed using ImageQuant software.

DNA Strand Annealing Assays—The DNA strand annealing activity of RECQ1 was measured using partially or fully complementary synthetic oligonucleotides (each at a concentration of 0.5 nM), one of which was labeled at the 5'-end using [γ -³²P]ATP and T4 polynucleotide kinase. Annealing of a ³²P-labeled oligonucleotide (0.5 nM) to M13mp18 ssDNA (1 nM) was also measured. Strand annealing reactions (20 μ l) were carried out in helicase reaction buffer (20 mM Tris-HCl (pH 7.5), 10 mM KCl, 8 mM dithiothreitol, 5 mM MgCl₂) and contained the indicated RECQ1 concentrations. Where specified, RPA (0.19–96 nM), *E. coli* single-stranded DNA-binding protein (0.75–768 nM), ATP (2 or 5 mM), ATP γ S (2 or 5 mM), and ADP (2 mM) were also present. Reactions were initiated by the addition of the unlabeled DNA strand, followed by incubation for 15 min at 37 °C. In kinetic experiments, 200- μ l reactions were initiated, and 20- μ l aliquots were removed at 0.5, 1, 2, 4, 8, 16, and 32 min. A control reaction mixture was set up in an identical manner to determine the level of spontaneous annealing of oligonucleotides in the absence of RECQ1. Reactions were terminated by the addition of 20 μ l of stop buffer. Reaction products were subsequently incubated with 0.1 mg/ml proteinase K for 15 min at 37 °C, and the products were resolved on native 12% polyacrylamide gels. Radiolabeled DNA species on polyacrylamide gels were visualized with a PhosphorImager and quantitated using ImageQuant software.

Immunodepletion—One microgram of rabbit anti-RECQ1 polyclonal antibody or normal rabbit IgG (both from Santa Cruz Biotechnology, Inc.) was incubated for 1 h at 4 °C with 40 μ l of protein G-agarose beads (Roche Applied Science) that had been equilibrated in helicase reaction buffer (see above). Beads were subsequently washed three times with helicase reaction buffer. Antibody-bound beads were then incubated with 20 nM RECQ1 in helicase reaction buffer (40 μ l) for 1 h at 4 °C. Beads were collected by centrifugation, and the supernatant was again incubated with antibody-bound beads for 10 min at 4 °C for a second immunodepletion step. Following centrifugation, supernatants were collected and divided into two equal aliquots, one to be assayed for strand annealing activity as described above and the other to assess the efficiency of RECQ1 depletion by Western blot analysis. In control experiments, protein G-agarose beads lacking any bound antibody were incubated either with or without RECQ1 (20 nM), and the resulting supernatants were tested for strand annealing.

Partial Proteolytic Digestion of RECQ1—For the limited proteolysis studies, purified recombinant RECQ1 (2 μ g) was incubated with chymotrypsin (0.1 μ g; Sigma) in the presence or absence of Mg²⁺ (3 mM) and ATP or ATP γ S (3 mM) in a 20- μ l reaction mixture at 37 °C for 45 min. An equal volume of 2 \times SDS sample buffer (126 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% bromophenol blue, and 10% β -mercaptoethanol) was added to the proteolytic digestion mixture, and samples were boiled for 5 min. Proteins were resolved on denaturing SDS-10% polyacrylamide gels and visualized by Coomassie Blue staining.

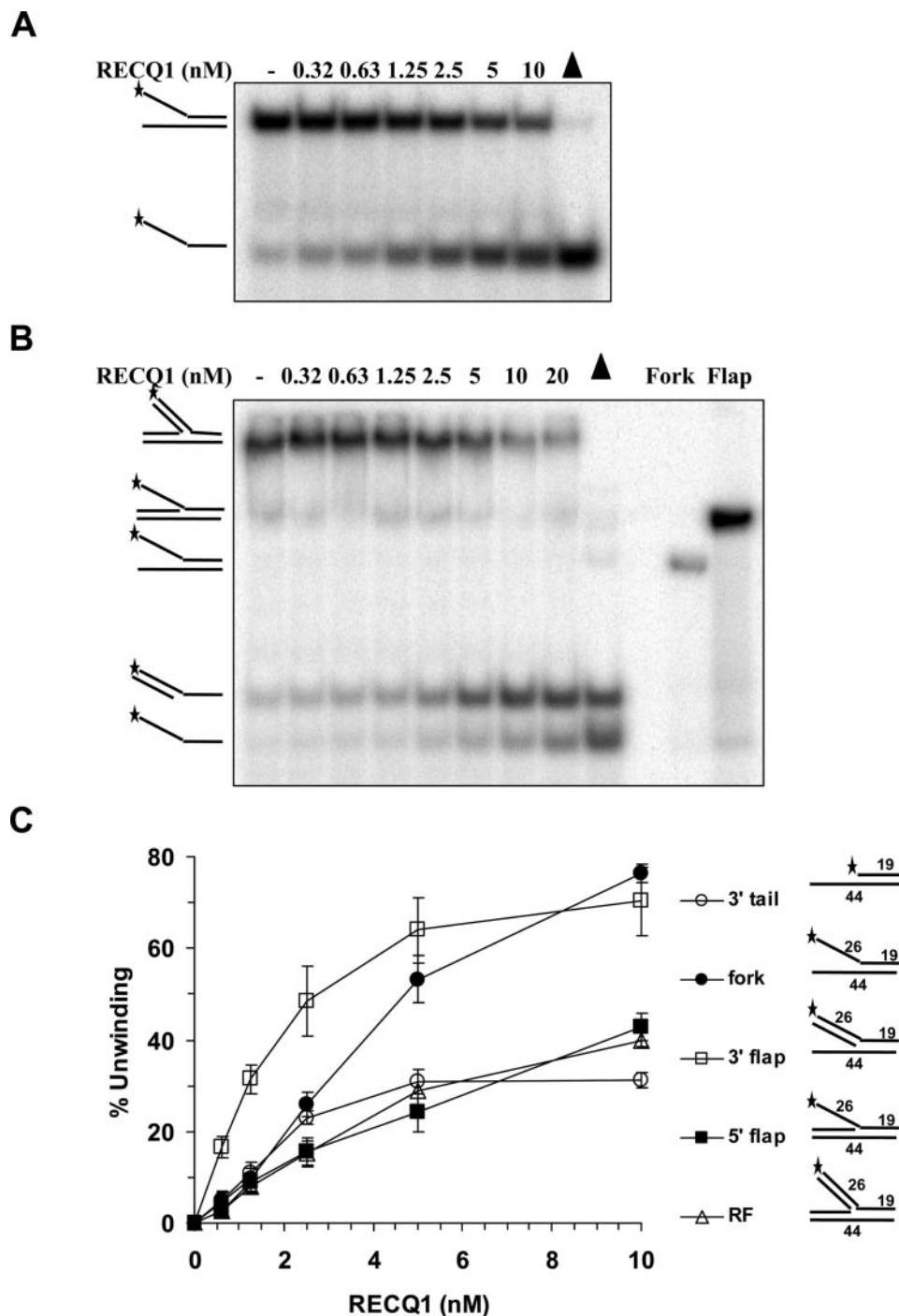


FIG. 1. **DNA substrate specificity of human RECQ1 helicase.** Helicase reactions were performed by incubating increasing RECQ1 concentrations with the indicated radiolabeled DNA substrate (0.5 nM) in 20 mM Tris-HCl (pH 7.5), 10 mM KCl, 8 mM dithiothreitol, 5 mM MgCl₂, 5 mM ATP, 10% glycerol, and 80 μg/ml bovine serum albumin. Reaction mixtures (20 μl) were incubated for 15 min at 37 °C, followed by proteinase K treatment, and subsequently loaded onto native 12% polyacrylamide gels. *A*, unwinding of 19-bp forked duplex DNA by RECQ1. ▲, heat-denatured DNA substrate control. *B*, unwinding of a synthetic replication fork structure by RECQ1. *C*, quantitative comparison of RECQ1 helicase activity on a series of related duplex DNA structures. The results shown are the average of at least three independent experiments, with S.D. indicated by error bars. *RF*, replication fork.

RESULTS

Although the effect of duplex length on RECQ1 helicase activity has been determined (15), we characterized the unwinding activity of purified recombinant RECQ1 helicase on a variety of DNA substrates that are proposed intermediates of various pathways of cellular DNA metabolism. In addition, we characterized how RECQ1 acts upon unwound ssDNA molecules to convert them to duplex strands by a strand annealing activity.

DNA Substrate Specificity of RECQ1 Helicase—To determine the DNA substrate preference for RECQ1 helicase activity, we examined a series of B-form duplex DNA substrates with a variety of combinations of ssDNA or dsDNA tails flanking the 19-bp duplex region. These DNA substrates, characterized by a related nucleotide sequence, were incubated with increasing concentrations of purified recombinant RECQ1 helicase in the presence of ATP, and the reaction products were analyzed on native polyacrylamide gels. The results demon-

strate that RECQ1 unwound these substrates to different extents depending on the presence and nature of the 3'- and 5'-tails (Fig. 1). Of the substrates tested, a forked duplex with 5'- and 3'-ssDNA arms of 26 and 25 nucleotides, respectively, was unwound relatively efficiently (Fig. 1, A and C). As expected from the unwinding polarity of RECQ1 (15), a DNA substrate flanked by only a 3'-ssDNA tail was also unwound by RECQ1; however, significantly less unwinding of this substrate was detected compared with the forked duplex with 3'- and 5'-ssDNA arms throughout the RECQ1 protein titration. The greatest difference (2.5-fold) was observed at a RECQ1 concentration of 10 nM (Fig. 1C).

The observation that RECQ1 unwound a forked DNA duplex with non-complementary 3'- and 5'-ssDNA tails significantly better than the simple 3'-ssDNA tailed duplex suggested that RECQ1, like WRN and BLM (18, 19), might unwind other DNA structures with junctions. Forked DNA substrates with either one or both of the arms in the double-stranded state are intermediates of cellular processes, including DNA replication, repair, and recombination. We therefore tested this substrate class, which includes a 3'-flap, a 5'-flap, and a synthetic replication fork with 3'- and 5'-duplex arms. Interestingly, the 3'-flap substrate was unwound the best of all the substrates tested. A significantly greater percent of the 3'-flap substrate (as much as 3.5-fold) was unwound by RECQ1 compared with the forked duplex with 3'- and 5'-ssDNA arms at the lowest RECQ1 protein concentrations tested (0.6, 1.2, and 2.5 nM) (Fig. 1C). The 5'-flap substrate was also unwound by RECQ1, but not nearly to the extent as either the forked duplex with ssDNA arms or the 3'-flap substrate. Despite the absence of a 3'-ssDNA tail, RECQ1 unwound the 5'-flap substrate as efficiently as the 3'-ssDNA tailed duplex, resulting in release of ~40% of the 5'-flap oligonucleotide at the highest RECQ1 concentration tested (10 nM) (Fig. 1C).

We next tested a forked duplex in which both the 5'- and 3'-tails of the fork were double-stranded. This three-way junction structure resembles a stalled replication fork where both the leading and lagging strands have been converted to duplex DNA. As shown in Fig. 1B, the synthetic replication fork was unwound by RECQ1 in a protein concentration-dependent manner. Quantitative analysis demonstrated that nearly the same percent of the synthetic replication fork and 5'-flap substrate was unwound by RECQ1 at a given helicase concentration, suggesting that the enzyme is indifferent to the single- or double-stranded character of the 5'-arm of these substrates. Gel analysis of the unwound products of the RECQ1 helicase reaction with the synthetic replication fork revealed that the substrate was unwound in the direction of the fork, primarily yielding a product with the duplex arm intact (Fig. 1B). These results suggest that RECQ1 recognized and bound the junction and translocated in a 3' to 5' direction along the bottom strand toward the direction of the fork, thus generating the partial duplex product. This activity is similar to that observed for the WRN helicase (18), but is distinct from that of *Drosophila* RECQ5 β , which was shown to unwind the lagging strand duplex of the synthetic replication fork structure (20). Notable, the results suggest that RECQ1 unwinds conventional duplex DNA substrates that completely lack ssDNA character and that its unwinding properties have both similarities to and differences from the unwinding properties of other RecQ helicases (see "Discussion").

RECQ1 Helicase Activity Is Sensitive to Free Magnesium Cation—The overall duplex DNA unwinding reaction catalyzed by a helicase is an energy-requiring process, and helicases utilize energy from the hydrolysis of ATP or other NTPs, al-

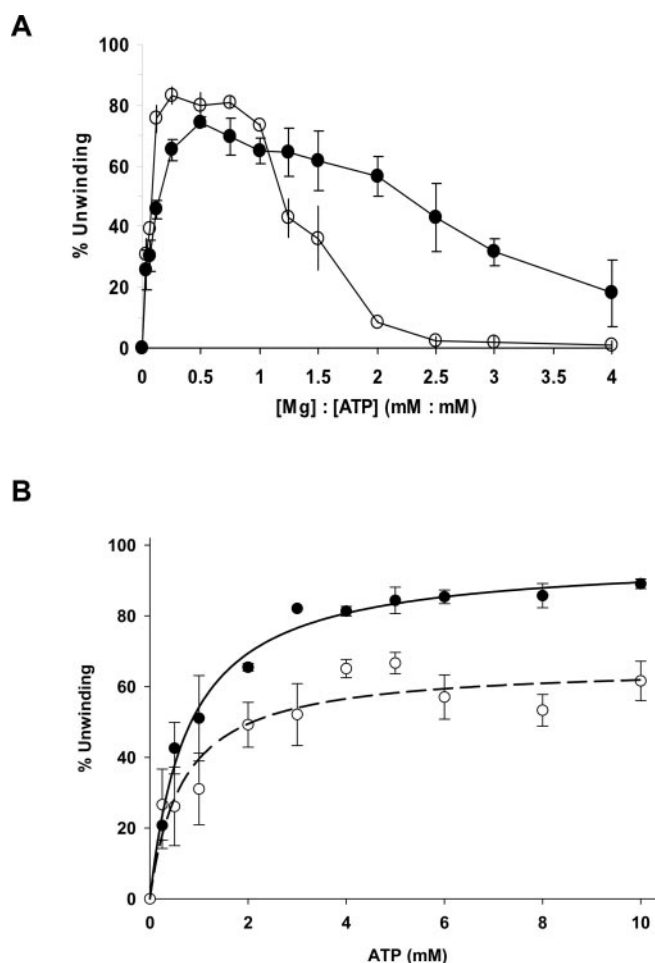


FIG. 2. Dependence of RECQ1 helicase activity on ATP and Mg^{2+} ion concentrations. A, RECQ1 helicase activity is inhibited by free Mg^{2+} ion concentration. Helicase reaction mixtures contained 0.5 nM forked DNA substrate, 10 nM RECQ1, and either 5 mM ATP with increasing concentrations of $MgCl_2$ from 0 to 20 mM (○) or 1 mM ATP with increasing concentrations of $MgCl_2$ from 0 to 4 mM (●). Percent unwinding in a 15-min reaction at 37 °C is shown. B, optimal ATP· Mg^{2+} complex concentration for RECQ1 helicase activity. Helicase reaction mixtures contained 0.5 nM forked DNA substrate, 10 nM RECQ1, and increasing concentrations of ATP· Mg^{2+} at a ratio of either 1:1 Mg^{2+} :ATP (○) or 0.5:1 Mg^{2+} :ATP (●). Percent unwinding in a 15-min reaction at 37 °C is shown. For A and B, helicase data (percent DNA substrate unwound) represent the mean of at least three independent experiments, with S.D. indicated by error bars.

lowing them to translocate and unwind DNA duplexes (1, 2). As the ATP· Mg^{2+} complex is the source of chemical energy for the RECQ1 helicase-catalyzed unwinding, we sought to determine the optimal concentrations of Mg^{2+} and ATP for RECQ1 helicase activity. Initially, we varied the Mg^{2+} concentrations at a fixed ATP concentration (5 or 1 mM) to estimate the ratio between ATP and Mg^{2+} at which the greatest level of RECQ1 unwinding could be detected and then to determine the optimal concentration of the ATP· Mg^{2+} complex. As shown in Fig. 2A, RECQ1 helicase activity in the presence of 5 mM ATP increased fairly linearly with Mg^{2+} :ATP ratios up to 0.5, but decreased with greater Mg^{2+} :ATP ratios, particularly evident at ratios >1. A steep decrease in RECQ1 helicase activity was observed at a Mg^{2+} :ATP ratio of 1.25, resulting in a 2-fold reduction in the percent of forked duplex substrate unwound. Additional increases in the Mg^{2+} :ATP ratio resulted in a greater reduction in unwinding by RECQ1. At a Mg^{2+} :ATP ratio of 2, only 10% of the substrate molecules were unwound by RECQ1 compared with 80% at a Mg^{2+} :ATP ratio of 0.5. To confirm the effect of free Mg^{2+} on RECQ1 helicase activity, we carried out the same

set of experiments using 1 mM ATP, which also resulted in a steady increase in RECQ1 helicase activity as a function of the Mg^{2+} :ATP ratio up to 0.5. Inhibition of RECQ1 helicase activity at Mg^{2+} :ATP ratios >0.5 was observed; however, the decrease in RECQ1 unwinding did not display as great a dependence on the Mg^{2+} :ATP ratio as observed at 5 mM ATP. These data indicate that RECQ1 helicase is sensitive to the ratio of Mg^{2+} to ATP, with maximal activity exhibited at Mg^{2+} :ATP ratios of ~ 0.5 – 1.0 . Beyond this optimum, the unwinding activity steadily declined with increasing Mg^{2+} :ATP ratios, suggesting that free Mg^{2+} ion is inhibitory to RECQ1 helicase activity. Sensitivity to free Mg^{2+} ion has also been reported for the *E. coli* RecQ helicase (21); however, WRN helicase activity, which displays optimal unwinding at an Mg^{2+} :ATP ratio of 1, is not adversely affected by greater Mg^{2+} :ATP ratios up to 4 (22).

We next performed experiments to determine the optimal concentration of the ATP- Mg^{2+} complex for RECQ1 helicase activity. Because RECQ1 is inhibited by free Mg^{2+} , RECQ1 helicase assays were performed in which the concentration of the ATP- Mg^{2+} complex (maintained at either a 1:1 or 0.5:1 ratio of Mg^{2+} to ATP) was increased. The percent of forked DNA substrate unwound was plotted as a function of ATP concentration (Fig. 2B). RECQ1 unwinding activity increased linearly with ATP concentration up to ~ 4 – 5 mM ATP, at which a plateau was approached for either fixed Mg^{2+} :ATP ratio (Fig. 2B). A Michaelis-Menten kinetic analysis of the initial hyperbolic regime of the plot shown in Fig. 2B yielded K_m and V_{max} values of 0.99 mM and 5.63×10^{-4} nm s $^{-1}$, respectively, for the 0.5:1 Mg^{2+} :ATP ratio. For the 1:1 Mg^{2+} :ATP ratio, the K_m and V_{max} values were 0.85 mM and 3.73×10^{-4} nm s $^{-1}$, respectively.

Kinetics of RECQ1 Helicase Activity—Having established the optimal concentrations of ATP and Mg^{2+} for the RECQ1 helicase reaction, we examined the kinetics of unwinding on a forked duplex DNA substrate with 5'- and 3'-ssDNA arms using different RECQ1 protein or DNA substrate concentrations. Using a fixed forked duplex substrate concentration of 0.8 nM, we determined that the amount of DNA substrate unwound by RECQ1 displayed a linear dependence with respect to time throughout the 180-s time course at helicase concentrations of 10 and 20 nM (Fig. 3A). The initial rate of fork unwinding was determined to be 0.0497 nM (bp)/s at a RECQ1 concentration of 10 nM, whereas 20 nM RECQ1 unwound the forked duplex with an initial rate of 0.0735 nM (bp)/s. Thus, the initial rates of unwinding were dependent on RECQ1 concentration.

We next examined the kinetics of RECQ1 helicase activity at a fixed RECQ1 concentration (20 nM), but at a 4-fold greater forked duplex substrate concentration (3.2 nM). As shown in Fig. 3B, the amount of DNA substrate (3.2 nM forked duplex molecules) unwound by RECQ1 exhibited linearity throughout the 180-s time course. For comparison, the time course in Fig. 3A using the same concentration of RECQ1 (20 nM) but only 0.8 nM forked duplex is shown in Fig. 3B. A quantitative comparison of the rates of RECQ1 helicase activity at the two DNA substrate concentrations indicated a 2.7-fold increase when 3.2 nM forked duplex substrate was present in the reaction compared with 0.8 nM. These kinetic results can be interpreted to mean that optimal RECQ1 catalytic activity was retained throughout the 3-min time course under these reaction conditions and that, at 0.8 nM DNA substrate and 20 nM RECQ1, the enzyme was not saturated with DNA substrate because a significantly greater rate of unwinding was observed when a 4-fold greater DNA substrate concentration was present in the reaction.

DNA Substrate Binding by RECQ1—Although, by their very nature, DNA helicases must load onto DNA substrates to ini-

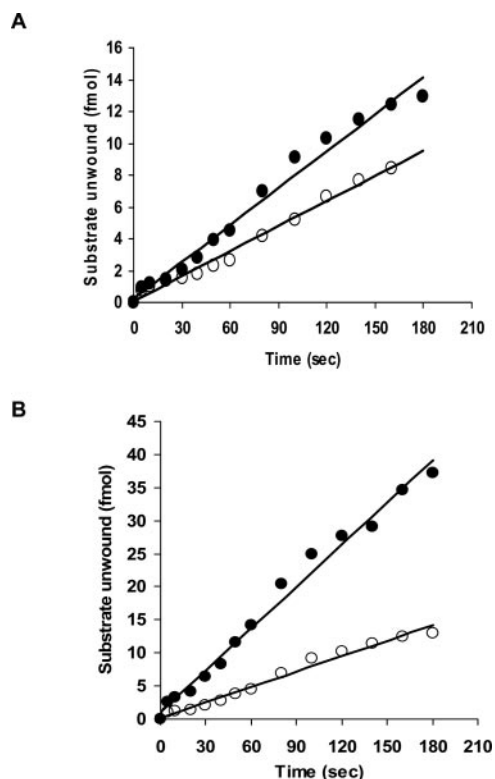


FIG. 3. Kinetic analyses of DNA unwinding by RECQ1 helicase. A, kinetics of RECQ1 unwinding as a function of protein concentration. Kinetic experiments were performed using the forked duplex DNA substrate (0.8 nM) at 10 nM (○) or 20 nM (●) RECQ1 as described under “Materials and Methods.” B, kinetics of DNA unwinding as a function of DNA substrate concentration. Kinetic experiments were performed using 20 nM RECQ1 and either 0.8 nM (○; data from A) or 3.2 nM (●) forked substrate as described under “Materials and Methods.” For A and B, helicase data (femtomoles of DNA substrate unwound) represent the mean of at least three independent experiments.

tiate unwinding, the relative abilities of RecQ helicases to stably bind DNA molecules vary. For example, *E. coli* RecQ binds to and unwinds a large variety of duplex DNA substrates, including blunt, ssDNA tailed, and forked duplex molecules (23). Sgs1 also binds a number of oligonucleotide-based duplex DNA molecules to varying extents (24). On the other hand, we have observed that, with the exception of the four-stranded HJ structure (25), full-length recombinant WRN helicase does not stably bind to various oligonucleotide duplex DNA substrates that the enzyme efficiently unwinds (18). Because the results presented here indicate that RECQ1 helicase has the ability to unwind a large variety of DNA structures, we wanted to characterize its physical interaction with DNA molecules to better understand its helicase substrate preference. Gel mobility shift assays were used to measure DNA binding by RECQ1 in the presence of the poorly hydrolyzable analog ATP γ S. RECQ1 effectively bound either the forked duplex or 3'-ssDNA tailed duplex substrate as evidenced by the three distinct slowly migrating species on native 5% polyacrylamide gels (Fig. 4, A and B, respectively). Similar patterns for gel-shifted species were observed for RECQ1 bound to ssDNA or blunt duplex DNA (data not shown). The shifted species may possibly reflect DNA molecules bound by distinct RECQ1 protein assembly states. Quantitative analysis of the DNA binding demonstrated that RECQ1 bound more efficiently to the forked duplex compared with the 3'-ssDNA tailed duplex (Fig. 4, A and B), suggesting that the preference of the RECQ1 helicase for the forked duplex substrate compared with the 3'-ssDNA tailed substrate may reflect its DNA substrate binding affinity.

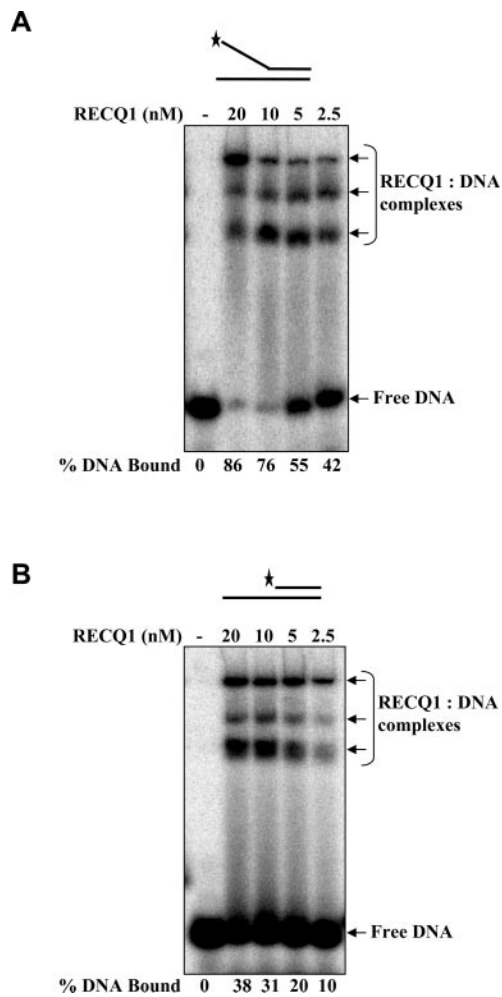


FIG. 4. RECQ1 binds DNA substrates as detected by gel electrophoresis mobility shift assays. Increasing RECQ1 concentrations were incubated with the indicated DNA substrate (0.5 nM) for 15 min on ice in DNA binding buffer. The protein-DNA complexes were then resolved on native 5% polyacrylamide gels. Radiolabeled bands were detected by PhosphorImager analysis. Typical gels of resolved RECQ1/DNA binding mixtures using the forked duplex (A) or 3'-ssDNA tailed duplex (B) are shown. Percent DNA substrate bound by RECQ1 is indicated below the lanes for each respective DNA binding mixture.

RECQ1 Promotes Branch Fork Migration of the Holliday Junction—The growing body of evidence suggests that RecQ helicases are involved in the replicational stress response by their roles in a non-recombinogenic pathway or RAD51-mediated homologous recombination (4, 26, 27). Localization of RecQ helicases (WRN, BLM, and Sgs1) at stalled DNA replication forks (25, 28, 29) and their ability to branch-migrate HJ intermediates (24, 25, 30) distinguish them from a number of other helicases involved in other aspects of DNA or RNA metabolism. To address a potential role of RECQ1 in branch fork migration, we tested its helicase activity on a synthetic four-stranded HJ structure. As shown in Fig. 5A, RECQ1 unwound the HJ(X12) DNA molecule in a protein concentration-dependent manner. Eighty-two percent of the HJ substrate was unwound by 10 nM RECQ1 in a 15-min reaction. In these reactions, the major product of HJ unwinding was a splayed arm product, suggesting that RECQ1 catalyzes disruption of the HJ by branch fork migration. To address this possibility, we examined the RECQ1 branch fork migration activity of the HJ substrate that had been preincubated with increasing concentrations of RuvA protein, an *E. coli* protein implicated in homologous recombination that has a very high affinity for the

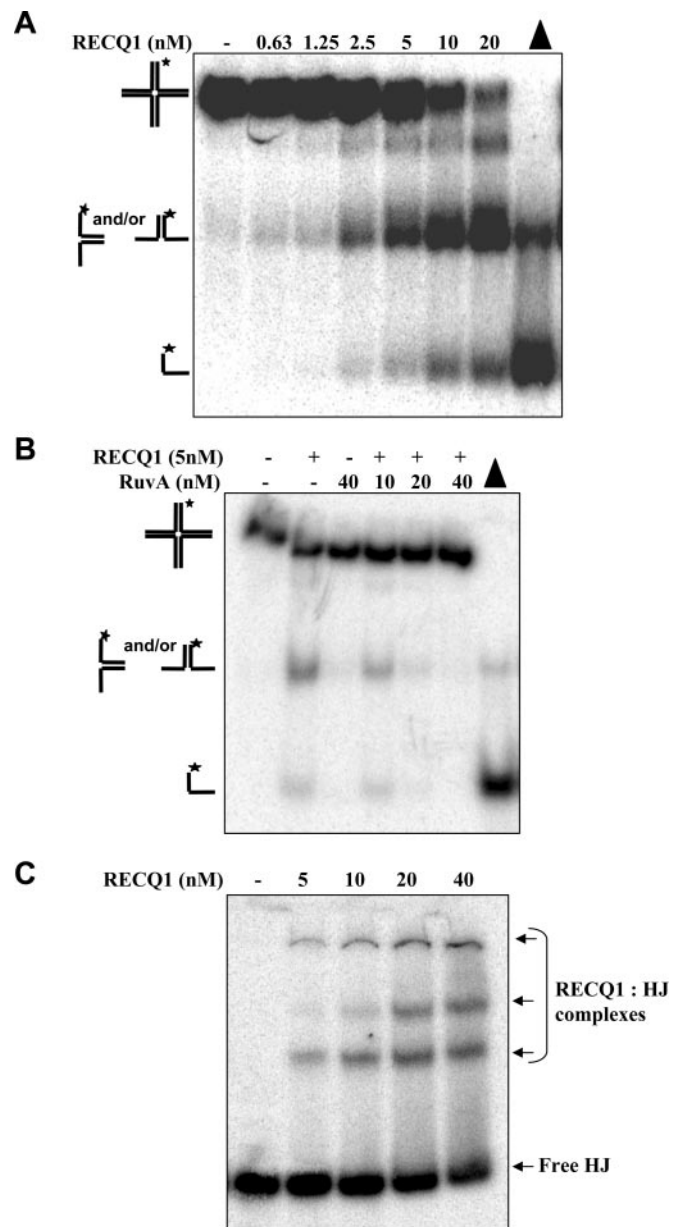


FIG. 5. Binding and unwinding of the HJ structure by RECQ1 helicase. A, RECQ1 catalyzes branch fork migration of the synthetic HJ(X12-1) substrate. Increasing concentrations of RECQ1 were incubated with 0.5 nM HJ(X12-1) under standard helicase reaction conditions for 15 min at 37 °C. Helicase reaction products were resolved on native 10% polyacrylamide gels. Forked duplex and 50-mer ssDNA products are indicated. ▲, heat-denatured DNA substrate control. B, RuvA inhibits RECQ1 unwinding of the HJ structure. Reaction mixtures containing 0.5 nM HJ(X12-1) were preincubated with the indicated concentrations of RuvA for 1 min at room temperature prior to the addition of RECQ1 (5 nM). Reactions were subsequently incubated for 15 min at 37 °C, and products were resolved as described for A. C, RECQ1 binds to the HJ substrate. Increasing concentrations of RECQ1 were incubated with 0.5 nM HJ(X12-1) in DNA binding buffer for 15 min on ice. Protein-DNA complexes were resolved on native 5% polyacrylamide gels.

HJ (31) and that blocks WRN branch migration activity on α -structures (25). RuvA inhibited RECQ1-catalyzed branch migration in a RuvA concentration-dependent manner (Fig. 5B). Consistent with its helicase activity on the HJ, RECQ1 bound the HJ substrate in a protein concentration-dependent manner as demonstrated by gel shift experiments (Fig. 5C). These results suggest that RECQ1 catalyzes branch migration of the synthetic HJ by initiating unwinding at the junction crossover.

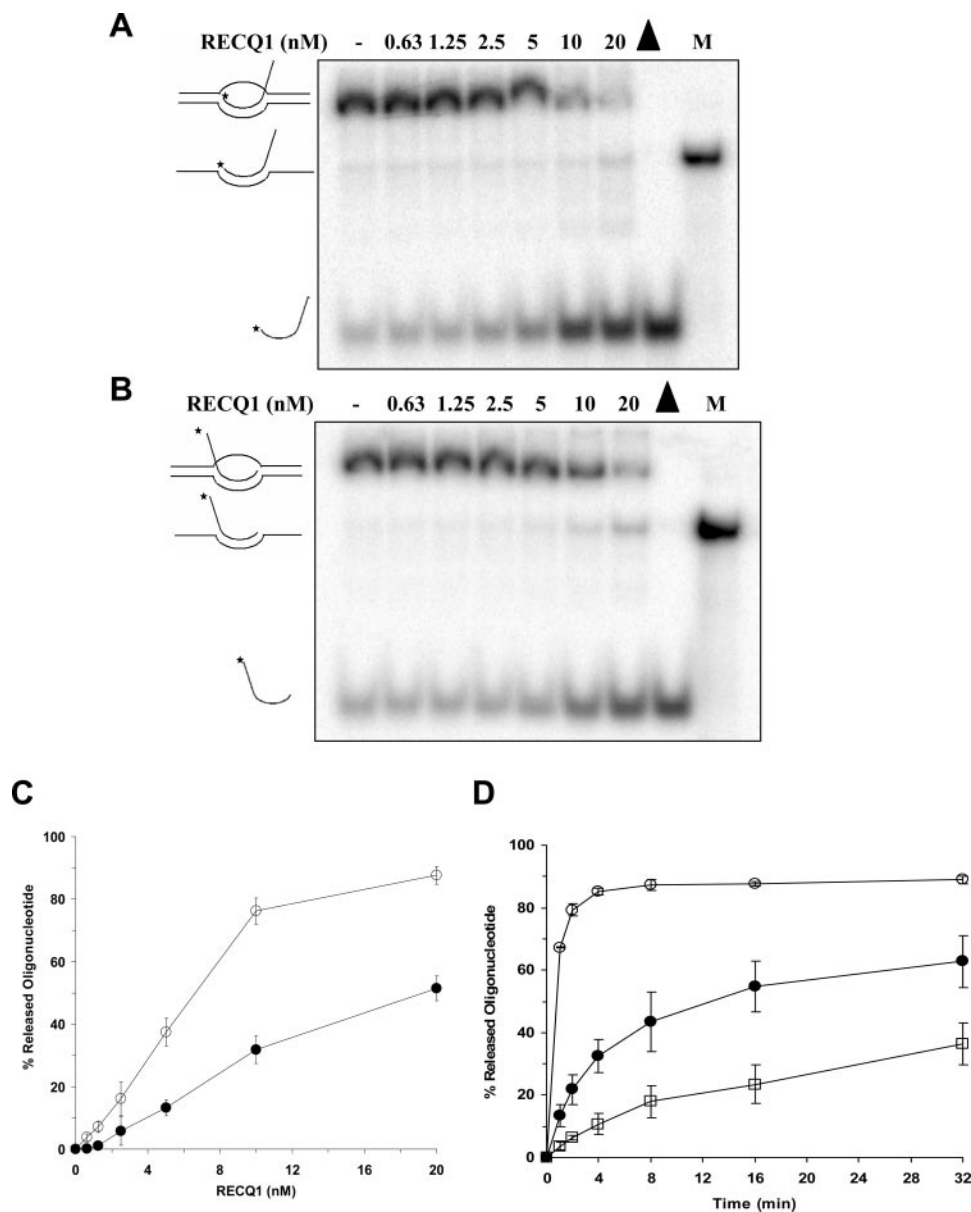
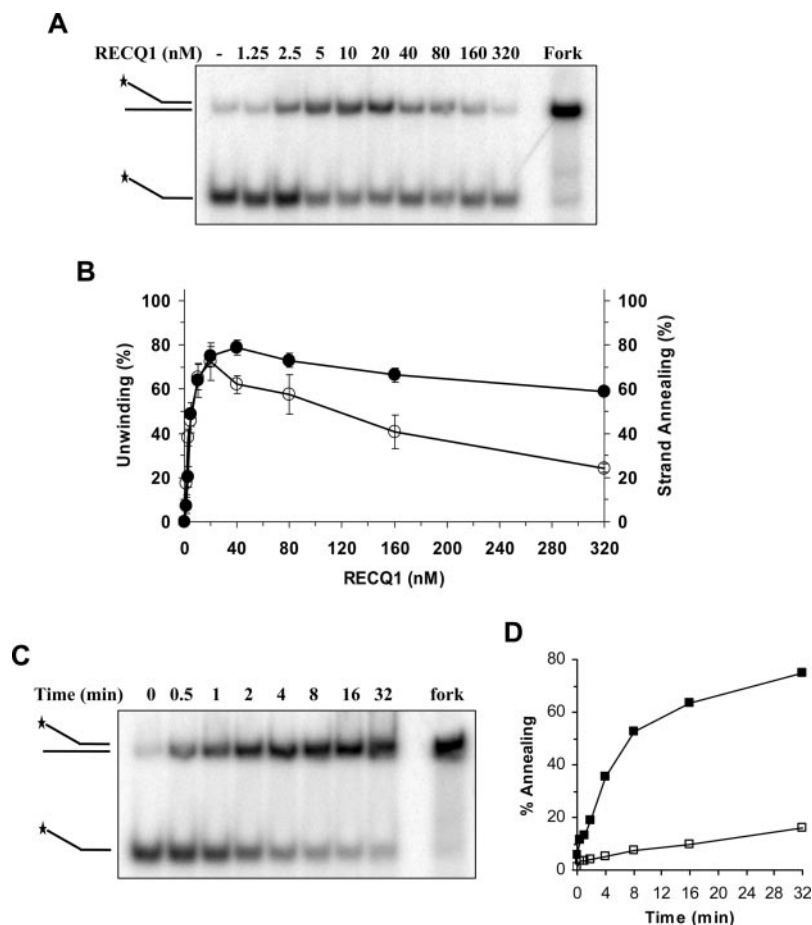


FIG. 6. **Melting of D-loops by RECQ1 helicase.** *A* and *B*, increasing concentrations of RECQ1 were incubated with 0.5 nM D-loop substrate with an invading third strand with a 3'-ssDNA or 5'-ssDNA tail, respectively, in standard helicase reaction salts containing 5 mM ATP for 15 min at 37 °C. Helicase reaction products were resolved on native 12% polyacrylamide gels. ▲, heat-denatured DNA substrate control; *M*, forked duplex marker. *C*, quantitative analyses of the RECQ1 unwinding activity of D-loop substrates with either a 3'-ssDNA (○) or 5' ssDNA (●) tail were performed. The results shown are the average of at least three independent experiments, with S.D. indicated by error bars. *D*, kinetic experiments were performed using 0.5 nM D-loop substrate with a 3'-ssDNA tail (○), a 5'-ssDNA tail (●), or no tail (flush; □) with 10 nM RECQ1 present as described under "Materials and Methods." Helicase data (percent oligonucleotide released) represent the mean of at least three independent experiments, with S.D. indicated by error bars.

RECQ1 Melts D-loops—The ability of RECQ1 to unwind branched DNA structures and to branch-migrate an HJ prompted us to examine whether RECQ1 helicase would metabolize other intermediates of homologous recombination. An early step in homologous recombination is the invasion of ssDNA into a homologous duplex to form a D-loop structure in which one strand of a duplex DNA molecule is displaced concomitant with formation of Watson-Crick base pairing between the complementary strand and an incoming single strand (32–34). To test RECQ1 activity on these early recombination intermediates, we prepared the D-loop substrates by annealing three oligonucleotides designed to form a single molecule (61 nucleotides in length) with a central non-complementary bubble of 21 residues flanked by 20 nucleotides of duplex to either side and in which the third strand invades and base pairs with

one of the looped-out strands in the 21-nucleotide bubble region (13). We first examined the effect of RECQ1 on D-loops with protruding single-stranded 5'- or 3'-tails of 20 residues extending from the invading third strand. In each case, the invading third strand was 5'-³²P-labeled so that dissociation of this strand could be monitored on native polyacrylamide gels. RECQ1 was able to unwind D-loops with either a protruding single-stranded 3'-tail (Fig. 6A) or 5'-tail (Fig. 6B) to release the invading third strand in a protein concentration-dependent manner; however, preferential unwinding of the D-loop with a protruding single-stranded 3'-tail was observed over the entire range of RECQ1 protein concentrations (Fig. 6, A–C). For example, at 2.5 nM RECQ1, ~16% of the invading third strand with a 3'-tail was released from the D-loop compared with only 6% of the 5'-tailed third strand (Fig. 6C); at 20 nM RECQ1, ~88

FIG. 7. RECQ1 catalyzes DNA strand annealing. *A*, assay of the strand annealing activity of RECQ1 by the formation of a 19-bp forked duplex in the presence of increasing concentrations of RECQ1. Two partially complementary oligonucleotides (0.5 nM each; one radiolabeled at the 5'-end and the other unlabeled) were incubated with the indicated concentrations of RECQ1 for 15 min at 37 °C in standard helicase reaction salts in the absence of ATP. The reaction products were resolved on native 12% polyacrylamide gels and detected by PhosphorImager analysis. The 19-bp forked duplex was loaded as a marker. *B*, RECQ1 mediates the protein concentration-dependent strand annealing of the two partially complementary oligonucleotides (○) or DNA unwinding of the forked duplex (●). ATP (5 mM) was present in the helicase reaction mixtures, but was absent from the strand annealing reaction mixtures. Quantitative analyses were performed. The results indicate the mean of at least three independent experiments, with S.D. indicated by error bars. *C*, kinetics of RECQ1-mediated strand annealing to form the 19-bp forked duplex. Kinetic experiments were performed using the partially complementary oligonucleotides (0.5 nM each) at 20 nM RECQ1. The 19-bp forked duplex was loaded as a marker. *D*, quantitative analyses of strand annealing kinetics to form the 19-bp forked duplex in the absence (□) or presence (■) of 20 nM RECQ1.



and 51% of the third strands were released from the D-loops with protruding single-stranded 3'- and 5'-tails, respectively (Fig. 6C).

We next performed kinetic analyses of D-loop unwinding by RECQ1. In addition to D-loops with protruding single-stranded 5'- or 3'-tails, we examined a D-loop structure with flush ends in which the third strand was completely complementary to the internal 21-nucleotide bubble region. Release of the invading third strand was monitored over a 32-min time course (Fig. 6D). RECQ1 (10 nM) displayed a strong preference for unwinding the D-loop with a protruding single-stranded 3'-tail compared with the D-loop substrates with a third strand with a 5'-ssDNA tail or no tail at all (Fig. 6D). Over a 32-min time course, RECQ1 catalyzed the release of 89, 63, or 37% of the invading third strand from the D-loop with a protruding single-stranded 3'-tail, a protruding single-stranded 5'-tail, or no tail, respectively (Fig. 6D). For comparison, we examined the ability of RECQ1 to unwind a bubble substrate in which the top and bottom strands were the same as those used for the creation of the three-stranded D-loop structures. Only 9% of the bubble was unwound by RECQ1 in 32 min (supplemental Fig. 1), suggesting that RECQ1 preferentially unwinds the three-stranded D-loop structures compared with the bubble substrate. Despite the observed differences in the rates of unwinding between the D-loop and bubble structures, RECQ1 was able to bind both classes of DNA molecules similarly as judged by gel mobility shift assays (supplemental Fig. 2). Thus, preferential unwinding of the D-loop substrate by RECQ1 is not determined solely by its DNA binding ability.

RECQ1 Catalyzes DNA Strand Annealing—Intermediates in homologous recombination are believed to involve multi-stranded branched recombinant DNA molecules that are a consequence of strand invasion and processing during HJ res-

olution. Our observations that RECQ1 has the ability to stably bind DNA molecules raised the possibility that its intrinsic DNA binding activity might be utilized for functions other than duplex DNA unwinding fueled by ATP hydrolysis. The recent report that human RECQ5 β catalyzes strand annealing (16) raised the possibility that RECQ1 might also perform this activity. Although other RecQ helicases have not been reported to catalyze strand annealing, specialization of human RecQ helicase function may be at least partly achieved by its coordinate balance between unwinding and strand annealing activities.

To investigate a putative RECQ1 strand annealing activity, we incubated two partially complementary single-strand oligonucleotides (0.5 nM each) with increasing concentrations of purified recombinant RECQ1 for 15 min and analyzed the products on native polyacrylamide gels. If strand annealing transpired, a 19-bp forked duplex would be formed. The results from a typical experiment demonstrate that RECQ1 promoted strand annealing of the two complementary strands to form the splayed arm duplex, which comigrated with the intact forked duplex control (Fig. 7A). RECQ1-promoted strand annealing was dependent on RECQ1 concentration, with increasing amounts of the annealed product being formed up to 20 nM RECQ1; however, RECQ1 concentrations >20 nM inhibited strand annealing (Fig. 7, A and B). For comparison, RECQ1 helicase activity on the forked duplex substrate over the same protein concentration range under the same reaction conditions (with the exception that 5 mM ATP was present in the helicase reactions) is shown in Fig. 7B. This comparison demonstrated that RECQ1-catalyzed strand annealing and helicase activities displayed a very similar dependence on RECQ1 concentration until the plateau was reached. These results suggest that RECQ1 has comparable ability to unwind the

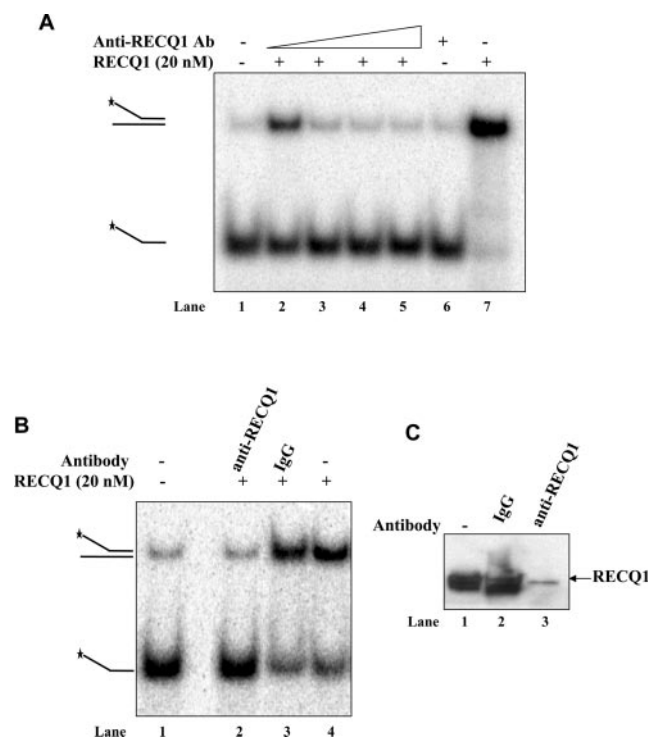


FIG. 8. RECQ1 immunodepletion inhibits RECQ1 strand annealing. *A*, RECQ1 was incubated with increasing amounts of anti-RECQ1 peptide antibody (*Ab*; lanes 2–5) for 5 min at 4 °C and was subsequently assayed for strand annealing activity by the formation of a 19-bp forked duplex as described under “Materials and Methods.” Lane 1, no-enzyme control; lane 6, antibody-alone control; lane 7, strand annealing promoted by RECQ1 not pretreated with antibody. *B* and *C*, rabbit anti-RECQ1 polyclonal antibody or normal rabbit IgG immobilized on protein G-agarose was used to immunodeplete RECQ1 as described under “Materials and Methods.” Half of the supernatant from each immunodepleted sample was assayed for strand annealing (*B*), and the other half was utilized for Western blot detection of RECQ1 (*C*) as described under “Materials and Methods.” *B*, strand annealing by RECQ1 in samples immunodepleted with anti-RECQ1 antibody (lane 2), rabbit IgG (lane 3), or no antibody (lane 4). Lane 1, no-enzyme control. *C*, Western blot showing RECQ1 in immunodepleted samples used for the strand annealing assay. Lane 1, RECQ1 incubated with protein G-agarose beads lacking any antibody; lane 2, immunodepletion with rabbit IgG-bound beads; lane 3, immunodepletion with anti-RECQ1 antibody-bound beads.

forked duplex substrate (in the presence of ATP) or to catalyze annealing of complementary single strands at low RECQ1 concentrations. A kinetic analysis demonstrated that strand annealing catalyzed by an optimal RECQ1 concentration (20 nM) displayed an initial linear rate during the first 8 min of the reaction, resulting in ~52% of the ssDNA being converted to the forked duplex (Fig. 7C). RECQ1 promoted annealing of 75% of the single-stranded molecules by 32 min compared with the ~16% spontaneous annealing that occurred in the absence of RECQ1 (Fig. 7D).

To address whether RECQ1 is responsible for the observed strand annealing activity, we examined the effect of anti-RECQ1 antibody on RECQ1-catalyzed strand annealing. Preincubation of RECQ1 with increasing amounts of anti-RECQ1 peptide antibody (14) resulted in the inhibition of RECQ1 strand annealing (Fig. 8A, lanes 2–5 versus lane 7). A similar inhibition was observed using an antibody directed against the N terminus of RECQ1 (data not shown). To address the specificity of inhibition of strand annealing by RECQ1, we tested the effect of either anti-RECQ1 antibody in reactions containing recombinant BLM protein, which catalyzes strand annealing.²

Both anti-RECQ1 antibodies did not inhibit strand annealing catalyzed by BLM (data not shown). To provide additional evidence that RECQ1 is responsible for the observed strand annealing, immunodepletion experiments were performed. In these studies, RECQ1 was immunodepleted prior to the strand annealing reaction. As shown in Fig. 8B, RECQ1 strand annealing was greatly reduced in the reaction that was immunodepleted for RECQ1 (lane 2 versus lane 4). Western blot analysis confirmed that RECQ1 protein was significantly depleted by pretreatment with anti-RECQ1 antibody (Fig. 8C, lane 3). In control reactions, preincubation of RECQ1 protein with normal IgG did not have an inhibitory effect on RECQ1 strand annealing (Fig. 8B, lane 3) and failed to immunodeplete RECQ1 (Fig. 8C, lane 2). These results provide evidence that RECQ1 is responsible for catalyzing strand annealing because the activity was inhibited in a specific manner by two RECQ1-directed antibodies, one against a unique peptide sequence and the other against the N terminus of RECQ1.

We next tested the ability of RECQ1 to catalyze strand annealing of a 100-mer to a target sequence in the M13mp18 ssDNA circular molecule. Although RECQ1 promoted annealing of the 100-mer to the M13mp18 ssDNA, a higher RECQ1 protein concentration was required to obtain the 100-bp M13 partial duplex product, and the yield of the annealed product was very low (~14% product formed by 160 nM RECQ1 in a 15-min assay) (supplemental Fig. 3). These results suggest that RECQ1-promoted strand annealing is compromised by the presence of large heterologous sequence in the DNA substrate and is dependent on DNA homology.

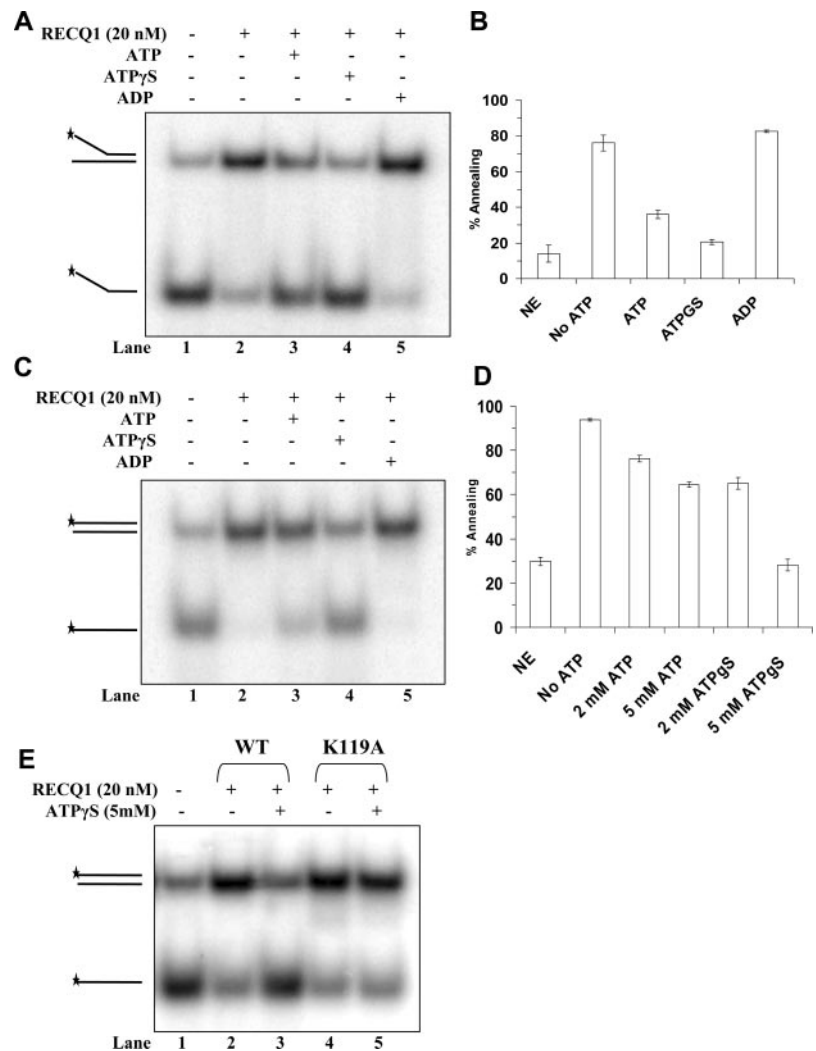
Modulation of RECQ1 Strand Annealing by Nucleotide Status—The ability of RECQ1 to catalyze DNA unwinding or strand annealing depending on ATP status suggested that the strand annealing activity might be regulated by nucleotide binding and/or hydrolysis. To address this issue, RECQ1 strand annealing was performed in the presence of 2 mM ATP, ADP, or ATP γ S (a poorly hydrolyzable ATP analog). At the optimal strand annealing concentration of 20 nM, RECQ1 converted 76% of the partially complementary single-strand oligonucleotide (0.5 nM) to forked duplex product in the absence of any nucleotide (Fig. 9, A, lane 2; and B). In contrast, ~36% annealed product was obtained in the presence of ATP (Fig. 9, A, lane 3; and B). The presence of ATP γ S inhibited strand annealing further to ~20% (Fig. 9, A, lane 4; and B), whereas the presence of ADP did not have any significant negative effect on RECQ1 strand annealing (A, lane 5; and B).

As the forked duplex product of the strand annealing reaction was a very good substrate for RECQ1 helicase activity, it was possible that the observed inhibition of RECQ1-mediated strand annealing in the presence of ATP was due to a competition between the helicase and strand annealing activities of the protein. Therefore, we performed similar experiments using complementary single-strand 44-mer oligonucleotides that will generate a 44-bp blunt duplex product upon annealing that will not be unwound by RECQ1 helicase in the presence of ATP. RECQ1 (20 nM) catalyzed efficient annealing of the complementary single strands such that 93% of the complementary single-strand oligonucleotides were converted to blunt duplex product in the absence of ATP (Fig. 9C, lane 2). The presence of 2 mM ATP resulted in a 17% reduction in RECQ1-mediated strand annealing (Fig. 9C, lane 3), whereas a 28% reduction was obtained when strand annealing was performed in the presence of 2 mM ATP γ S (lane 4). ADP (2 mM) did not affect RECQ1-mediated strand annealing (Fig. 9C, lane 5).

Because recombinant RECQ1 has been shown to be a DNA-dependent ATPase (14) and could also hydrolyze ATP in the

² C. F. Cheek and I. D. Hickson, personal communication.

FIG. 9. ATP binding inhibits RECQ1-mediated strand annealing. A, the effect of ATP, ATP γ S, or ADP on RECQ1 (20 nM)-mediated strand annealing was assayed by the formation of the 19-bp forked duplex under standard strand annealing conditions in the absence or presence of the indicated nucleotide (2 mM). B, the results from the experiments as shown in A were quantitated. NE, no-enzyme control. C, the effect of ATP, ATP γ S, or ADP on RECQ1 (20 nM)-mediated strand annealing was assayed by the formation of a 44-bp blunt duplex under standard strand annealing conditions in the absence or presence of the indicated nucleotide (2 mM). D, the results from the experiments using ATP or ATP γ S at the indicated concentrations were quantitated. The results shown in B and D are the mean of at least three independent experiments, with S.D. indicated by error bars. E, the effect of ATP γ S on strand annealing mediated by the RECQ1-K119A mutant (20 nM) or wild-type (WT) RECQ1 (20 nM) was assayed by the formation of a 44-bp blunt duplex under standard strand annealing conditions in the absence or presence of ATP γ S (5 mM).



presence of double-stranded DNA,³ it was conceivable that ATP turnover plays a role in the modulation of RECQ1 strand annealing. To address this, RECQ1 strand annealing to form the blunt duplex DNA product was conducted in the presence of 5 mM ATP or ATP γ S. A slight but statistically significant greater inhibition of strand annealing was observed at 5 mM ATP compared with 2 mM ATP (Fig. 9, C and D). However, significantly greater inhibition of strand annealing was detected at 5 mM ATP γ S compared with 2 mM ATP γ S. In fact, the level of RECQ1 strand annealing in the presence of 5 mM ATP γ S was comparable with the level of spontaneous strand annealing (29%) (Fig. 9, C and D). These results suggest that ATP binding by RECQ1 is inhibitory to its strand annealing activity.

To gain further insight into the effect of ATP on RECQ1 strand annealing, we tested a mutant recombinant RECQ1 protein containing a site-specific replacement of the conserved lysine residue of the Walker A box (motif I) implicated in ATP binding with an alanine residue (RECQ1-K119A). The K119A mutation abolished RECQ1 helicase activity on the same forked duplex substrate used in this study (17) and also eliminated RECQ1 ATPase activity (data not shown). As shown in Fig. 9E, the mutant RECQ1-K119A protein retained its ability to catalyze strand annealing comparable with the wild-type RECQ1 protein. However, in the presence of ATP γ S, RECQ1-

K119A, unlike wild-type RECQ1, retained its ability to catalyze strand annealing. These results provide additional evidence that the observed strand annealing activity is intrinsic to RECQ1 and support our findings that ATP binding by RECQ1 alters its ability to perform strand annealing.

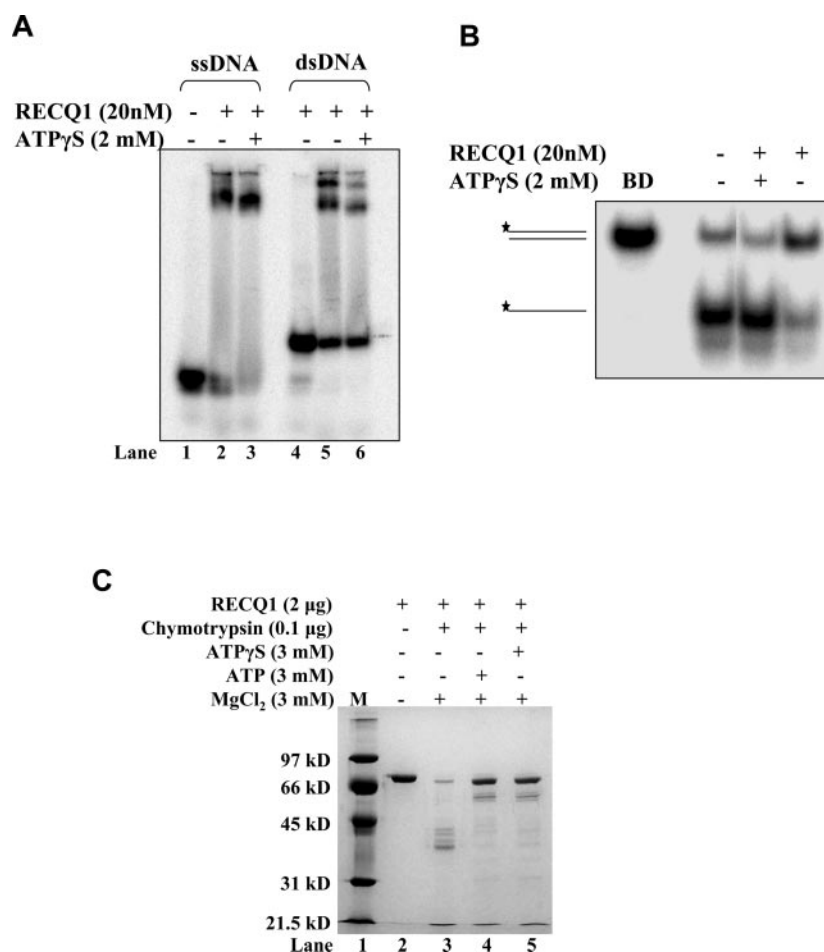
We next examined whether ATP γ S modulates RECQ1 binding to ssDNA or dsDNA to determine whether this is a component of the nucleotide inhibition of RECQ1 strand annealing. RECQ1 bound efficiently to a single-stranded 44-mer or a 44-bp duplex in the presence or absence of 2 mM ATP γ S (Fig. 10A). Under conditions in which RECQ1 was prebound separately to the two complementary oligonucleotides, significantly reduced strand annealing was observed when ATP γ S was present (Fig. 10B), as observed previously (Fig. 9C). These results suggest that the inhibition of strand annealing by ATP-bound RECQ1 is not due to modulation of the DNA binding activity of RECQ1.

Nucleotide-induced Conformational Change in RECQ1 Protein—We next sought to determine whether ATP binding induces a conformational change in RECQ1 protein that might be responsible for its effect on RECQ1 strand annealing activity. Chymotrypsin was used for the partial proteolysis of RECQ1 in the presence or absence of ATP or ATP γ S (Fig. 10C). RECQ1 was digested by chymotrypsin in a 45-min reaction, yielding a characteristic pattern of RECQ1 proteolytic fragments (Fig. 10C, lane 3). The presence of ATP or ATP γ S in the chymotrypsin digestion reaction altered the proteolysis pattern of RECQ1 and rendered the protein partially resistant to digestion by chymotrypsin (Fig. 10C, lanes 4 and 5). Similar results were

³ S. Sharma, J. A. Sommers, S. Choudhary, J. K. Faulkner, S. Cui, L. Andreoli, L. Muzzolini, A. Vindigni, and R. M. Brosh, Jr., unpublished data.

FIG. 10. ATP binding that induces a conformational change in RECQ1 protein does not alter its DNA binding.

A, RECQ1 binding to the labeled 44-mer or 44-bp blunt duplex in the presence or absence of ATP γ S (2 mM) in standard strand annealing buffer (see "Materials and Methods") was analyzed by gel mobility shift assay. RECQ1 was incubated with DNA for 10 min at room temperature. **B**, in a 10- μ l volume, RECQ1 was incubated separately with either a 32 P-5'-end-labeled 44-mer or its complementary unlabeled 44-mer for 10 min at room temperature in standard strand annealing buffer. The contents of the two tubes were subsequently mixed together in the presence or absence of 2 mM ATP γ S, and the mixture was then incubated for 15 min at 37 °C. The samples were treated with proteinase K, and the DNA was resolved on native 12% polyacrylamide gels to monitor strand annealing by the formation of the 44-bp blunt duplex from the complementary 44-mers. *First lane*, blunt duplex (BD) marker; *second lane*, spontaneous strand annealing in the absence of RECQ1; *third and fourth lanes*, strand annealing of 44-mers preincubated with RECQ1 in the presence and absence of 2 mM ATP γ S, respectively. **C**, shown are the effects of ATP or ATP γ S on partial proteolytic digestion of RECQ1 by chymotrypsin. RECQ1 (2 μ g) was incubated with 0.1 μ g of chymotrypsin (enzyme:substrate ratio of 1:20) in the presence or absence of Mg $^{2+}$ (3 mM) and ATP or ATP γ S (3 mM) in a 20- μ l reaction mixture at 37 °C for 45 min. The protein samples were resolved on denaturing SDS-10% polyacrylamide gels and stained with Coomassie Blue. *M*, protein molecular mass markers.



obtained when the proteolysis experiments were performed in the presence of ssDNA (data not shown). These results suggest that ATP binding induces conformational change(s) in RECQ1 protein that leads to its protection by chymotrypsin proteolysis.

Effect of RPA on RECQ1-mediated Strand Annealing—Human RECQ1 helicase has been shown to physically and functionally interact with the ssDNA-binding protein RPA (14). To test whether the strand annealing activity of RECQ1 protein is modulated by RPA, we conducted RECQ1 strand annealing assays in the presence of increasing RPA concentrations. As shown in Fig. 11, RECQ1 strand annealing was inhibited by RPA in an RPA concentration-dependent manner. Strand annealing by RECQ1 (20 nM) was reduced from 65% (Fig. 11A, lane 2; and B) to 42% (A, lane 7; and B) by 3 nM RPA. Taking into account that there are approximately two RPA-binding sites/44-mer, 34% inhibition was observed at an *R* value (ratio of ssDNA-binding protein-binding units to DNA-binding site) of 1.5 (i.e. at 3 nM RPA) (Fig. 11, A, lane 7; and B) and 90% inhibition at an *R* value of 3 (6 nM RPA) (A, lane 8; and B). An additional increase in RPA concentration to 12 nM resulted in complete inhibition of RECQ1 strand annealing (Fig. 11, A, lanes 8–12; and B). Inhibition of RECQ1 strand annealing by the *E. coli* ssDNA-binding protein was also observed at similar *R* values (data not shown), suggesting that the inhibition was mediated by DNA interactions rather than specific protein interactions.

DISCUSSION

Within the last decade, a plethora of research has implicated RecQ helicases as important mediators of genomic stability maintenance by their cellular roles in pathways that deal with replicational stress and/or DNA damage. Understanding the

cellular mechanisms by which RecQ helicases perform their vital functions has gained prominence since the discovery that several premature aging diseases and cancers are genetically linked to deficiencies in human RecQ helicases. Although progress has been made in understanding the molecular, cellular, and genetic functions of the WRN and BLM helicases, less is known about RECQ4 (defective in Rothmund-Thomson syndrome (35), but its gene product lacks helicase activity (36)), RECQ1 (the first human RecQ helicase to be identified (37, 38)), and RECQ5 (very recently shown to catalyze DNA unwinding and strand annealing (16)).

In this study, we have performed extensive biochemical characterization of purified recombinant human RECQ1 protein. We have characterized the preference of RECQ1 to unwind a variety of duplex DNA substrates. In addition to more standard duplex substrates flanked by either a 3'-ssDNA tail or both 3'- and 5'-ssDNA tails, RECQ1 was shown to unwind 3'-flap, 5'-flap, and synthetic replication fork structures. Although all RecQ helicases characterized to date exhibit 3' to 5' polarity of unwinding with respect to the strand that the helicase is presumed to translocate, differences in the substrate preference and/or *directionality* of unwinding on replication/repair DNA intermediates exist. For example, *Drosophila* RECQ5 β unwinds the lagging strand of a synthetic replication fork (20); in contrast, RECQ1, like WRN (18), unwinds the replication fork structure in the direction of the fork, suggesting some apparent differences among the RecQ helicases. This leads to the hypothesis that, because the disease states and cellular phenotypes of *recQ* mutants are distinguishable from each other, the molecular functions of the RecQ helicases themselves must be discernible.

A

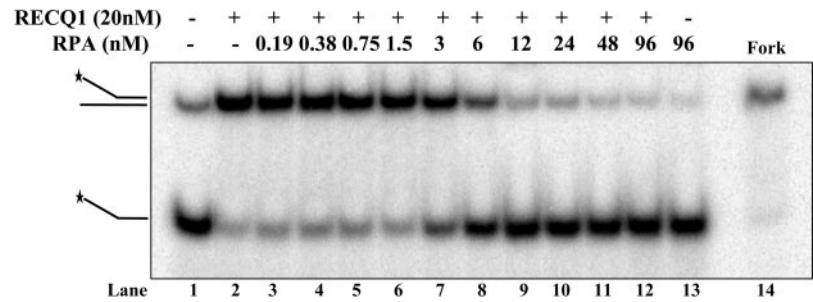
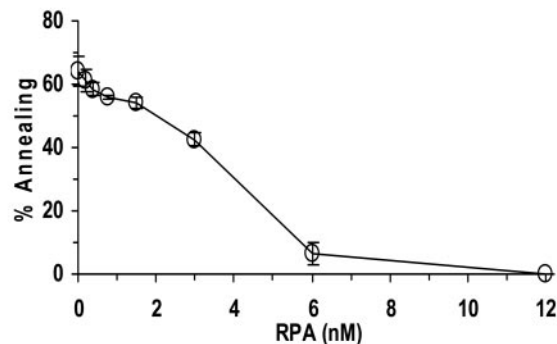


FIG. 11. RPA inhibits RECQ1-mediated strand annealing. A, strand annealing in the presence of 20 nM RECQ1 was assayed by the formation of the 19-bp forked duplex from the partially complementary 44-mers in the absence and presence of the indicated concentrations of RPA. The labeled 44-mer and unlabeled complementary oligonucleotides (0.5 nM each) were incubated for 5 min at 37 °C with the indicated amounts of RPA in annealing reaction mixtures, and reactions were initiated by the addition of RECQ1 (20 nM), followed by a 15-min incubation at 37 °C. Reaction products were resolved on native 12% polyacrylamide gels and analyzed using a PhosphorImager. The 19-bp forked duplex is shown as a marker. B, the results from the experiments shown in A were quantitated. The results are the average of at least three independent experiments, with S.D. indicated by error bars.

B



A common theme suggested for RecQ biology is that the genomic instability of *recQ* mutants may be a consequence of inappropriate processing of DNA replication or recombination intermediates that arise during conditions of replicational stress (9, 27). If a replication fork stalls or arrests, homologous recombination pathways may be elicited to deal with the structural anomaly. A stalled replication fork can be converted to a four-way junction resembling an HJ by branch migration and re-annealing of nascent DNA strands (39, 40). Here, we have reported that RECQ1, like a number of other RecQ helicases (e.g. WRN, BLM, and Sgs1), branch-migrated a synthetic HJ structure to convert it to a splayed arm product in a reaction dependent on ATP hydrolysis. A potential role of RECQ1 or a related RecQ helicase in the rescue of a stalled fork is to catalyze reverse branch migration past the lesion to reset the replication fork, and the lesion can be subsequently corrected by DNA repair (41).

In addition to the HJ structure, RECQ1 unwinds another key intermediate of homologous recombination, the D-loop structure. The ability of RECQ1 to release the invading third strand from D-loop structures may be important as an anti-recombinase function, as proposed for other RecQ helicases (13, 42). Alternatively, efficient unwinding of D-loops by RECQ1 may play a role in telomere maintenance, as proposed for WRN helicase (43), or in DNA repair by a synthesis-dependent strand annealing pathway that requires multiple rounds of strand invasion/resynthesis for the complementary strand to anneal to its partner strand, as discussed below.

Certain biochemical properties of RECQ1 are not shared by other RecQ helicases. DNA unwinding catalyzed by RECQ1 is sensitive to free Mg^{2+} ion concentration, whereas WRN unwinding is resistant to Mg^{2+} :ATP ratios up to 4 (22). One notable biochemical property of RECQ1 is that the enzyme proficiently binds DNA. Although other RecQ helicases have also been shown to bind DNA, their abilities to bind various ssDNA or dsDNA molecules widely differ. For instance, whereas yeast Sgs1 and *E. coli* RecQ stably bind DNA (23, 24),

the interaction of WRN with standard duplex DNA molecules is fairly transient, as evidenced by the poor ability to trap protein-DNA complexes in gel mobility shift assays (18). The evidence presented here demonstrates that RECQ1 binds both ssDNA and dsDNA molecules, suggesting that the enzyme may utilize this property in ways that WRN does not.

The demonstrated ability of RECQ1 to stably bind DNA as well as to dimerize in solution (14) suggested to us that the protein may utilize its ssDNA binding ability to facilitate strand annealing between DNA molecules that could form complementary Watson-Crick base pairs. A biochemical analysis of human RECQ5 β demonstrated that this protein can promote strand annealing (16); however, an important distinction between RECQ1 and RECQ5 helicases is that RECQ1 efficiently unwinds duplex DNA substrates in the absence of any auxiliary factor, whereas RECQ5 β requires the presence of RPA to efficiently unwind DNA duplexes (16). Although the reaction conditions are slightly different, a comparison of our results with those of Janscak and co-workers (16) suggests that RECQ1 unwinds dsDNA more efficiently than RECQ5 β in the absence of RPA. Along these lines, the observation that RECQ4 fails to unwind DNA (36) suggests that other catalytic or non-catalytic functions of RecQ helicases are important for their biological roles. A catalytic function other than helicase activity that RECQ5 β and RECQ1 share is strand annealing. Despite the fact that both human RecQ proteins can catalyze DNA unwinding and strand annealing, the subtle differences in their enzymatic activities may contribute to their specialization.

Analyses of nucleotide effects on strand annealing revealed that ATP binding inhibits strand annealing promoted by either RECQ1 or RECQ5 β (16). In this study, we demonstrated that ATP binding by RECQ1 induced a conformational change in RECQ1 protein as detected by partial proteolysis studies. A nucleotide-induced conformational change in RECQ1 protein may alter its structure, suggesting a mechanism for the molecular switch from a DNA unwinding mode to a strand annealing mode. Although the assembly state for the actively unwinding

or strand annealing forms of RECQ1 is not known, the results from this work suggest that nucleotide-induced conformational changes that alter structure may be important in the transition from unwinding to strand annealing activities.

Some insight as to how the coordinate functions of DNA unwinding and strand annealing by the same protein are important in a single pathway may be garnered from a model proposed for the role of *Drosophila* BLM in the SDSA pathway during repair of double-strand breaks (11, 12). According to this model, the repair synthesis during SDSA is not processive, releasing the newly synthesized strand to search for a complementary sequence. In the absence of a complementary single strand, the nascent strand is further extended by a subsequent round of strand invasion and DNA synthesis. *In vitro*, human RECQ1 releases the invading third strand from D-loop structures, suggesting that the enzyme may act to release the newly synthesized strand from a RAD51-mediated D-loop formed *in vivo* during recombination or processing of replication intermediates. However, the ability of RECQ1 to catalyze DNA strand annealing could be utilized to anneal a newly synthesized strand to its complementary strand as the final step after repair synthesis. A role for BLM or RECQ1 in SDSA is relevant because most mitotic recombination events are suggested to proceed through SDSA. Unlike a homologous recombination pathway that involves HJ resolution to produce crossovers, repair of double-strand breaks by SDSA results in gene conversion without crossing over to preserve genomic integrity (44).

What evidence suggests that RECQ1 may have redundant functions with another RecQ helicase to maintain genomic stability? In a recent study, simultaneous deletion of RECQ1 and BLM in chicken DT40 cells resulted in slow growth, increased cell death, and higher incidence of sister chromatid exchange upon mitomycin C treatment (10). In contrast, RECQ5^{-/-} BLM^{-/-} cells exhibit elevated sister chromatid exchange in the absence of DNA damage (10). These results suggest that BLM, RECQ1, and RECQ5 may have partially redundant roles in maintaining genomic integrity, but their roles only overlap under certain circumstances *in vivo*. In the case of RECQ1 and BLM, the two proteins may substitute for each other when DNA damage blocks replication fork progression; however, RECQ1 does not perfectly substitute for BLM because BLM^{-/-} cells still display elevated sister chromatid exchange. Thus, the requirement for BLM is more stringent because RECQ1^{-/-} cells do not display a mutant phenotype. Nonetheless, our results demonstrating that RECQ1 not only efficiently unwinds D-loops but also catalyzes strand annealing are consistent with the notions that RECQ1 and BLM may have redundant functions in the SDSA pathway and that, in their dual absence, recombination intermediates persist that lead to an even greater sister chromatid exchange. Additional cellular and molecular studies of BLM and RECQ1 with their mutual protein interactors (*e.g.* topoisomerase III α) (45, 46) will create insights into the functional dynamics between the two human RecQ helicases.

Acknowledgments—We thank Dr. Ian Hickson (Cancer Research UK) for recombinant BLM protein. We thank members of the International Centre for Genetic Engineering and Biotechnology and the Laboratory of Molecular Gerontology (NIA, NIH) for helpful discussions.

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**Biochemical Analysis of the DNA Unwinding and Strand Annealing Activities
Catalyzed by Human RECQ1**

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J. Biol. Chem. 2005, 280:28072-28084.

doi: 10.1074/jbc.M500264200 originally published online May 16, 2005

Access the most updated version of this article at doi: [10.1074/jbc.M500264200](https://doi.org/10.1074/jbc.M500264200)

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