

Enzyme-catalyzed DNA Unwinding

MECHANISM OF ACTION OF HELICASE III*

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Helicase III isolated from *Escherichia coli* catalyzes the hydrolysis of ATP or dATP provided that single-stranded DNA is present. Single-stranded DNA can be rendered inactive as a cofactor if the DNA is first made to form a complex with *E. coli* DNA binding protein. We show that this result is due to the binding protein preventing attachment of helicase III to the DNA. The *rep* protein which is isolated from *E. coli* and is also a single-stranded DNA-dependent ATPase is likewise affected by the presence of *E. coli* DNA binding protein.

Helicase III and *rep* protein are each able to catalyze unwinding of duplex DNA in the presence of ATP and *E. coli* DNA binding protein. The *rep* protein shows an absolute requirement for *E. coli* DNA binding protein, whereas helicase III shows a 4-fold dependence. For each of the unwinding enzymes, the *E. coli* DNA binding protein can be replaced by the T4 helix-destabilizing protein. This suggests that the role of the binding protein is to keep the already separated DNA strands apart.

The *rep* protein and helicase III differ in one important respect with regard to the mechanism of duplex unwinding. The proteins appear to catalyze unwinding by moving along DNA with opposite polarities. Each protein attaches to a single-stranded protrusion and then invades the DNA duplex. The *rep* protein moves along the single-stranded tail in a 3' to 5' direction, whereas helicase III moves in a 5' to 3' direction. These results suggest that both proteins, each binding to opposite strands of DNA, can promote unidirectional unwinding at the replication fork during DNA replication.

Studies on the *rep* protein demonstrated that this protein unwinds DNA catalytically in an ATP- and DNA binding protein-dependent reaction (14). We proposed a model to explain the mechanism of ATP-dependent DNA unwinding in which one enzyme molecule could processively unwind a long duplex DNA molecule, provided that DNA binding protein acts to keep the unwound strands from renaturing. Since both helicase enzymes isolated from uninfected cells seem to act stoichiometrically, and since there is not an obligatory requirement for *rep* protein in cellular replication (18), we searched for another catalytically acting unwinding enzyme. The accompanying report (19) describes the purification and general properties of a novel DNA-dependent ATPase enzyme. This report analyzes in greater detail the catalysis of DNA strand separation and also compares this activity to the *rep* protein activity. We will demonstrate that both proteins catalyze strand separation; however, they appear to move along DNA in opposite directions. A model is presented in which both the *rep* protein and helicase III function together (each on a different parental DNA strand) to promote unidirectional replication fork movement.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were as described in the preceding paper.

Enzymes—DNA binding protein (*E. coli*) was purified to homogeneity according to our previous procedure (20) and was free of detectable exonuclease I and RNase H activities. T4 helix-destabilizing protein was a gift of Dr. B. Alberts, University of California, San Francisco, Medical School, and contained no detectable ATPase activity or measurable amounts of *E. coli* DNA binding protein. *Rep* protein (5×10^6 ATPase units/mg protein) was purified to the DNA cellulose stage, and *cisA* protein was purified to homogeneity as described before (14). Helicase III (1×10^6 ATPase units/mg protein) was purified as described in the preceding paper (19). Nuclease S1 was from Boehringer-Mannheim.

DNA Substrates—DNA substrates were all prepared by previously published methods (see preceding paper (19)). Partial duplex molecules for the study of DNA unwinding experiments were prepared exactly as described previously (14).

DNA Strand Separating Assay—The strand separation assay (0.1 ml) contained 20 mM Tris-HCl, pH 8.0, 4 mM dithiothreitol, 12 mM MgCl₂, 3 mM ATP, 1.5 nmol of DNA substrate, and 50 to 300 units of helicase III. DNA binding protein was added as indicated in the figure legends. After incubation for 20 min at 37°C, an equal volume of 4 M NaCl was added along with 20 µg of denatured calf thymus DNA. Following a 5-min incubation at 37°C, the reaction mixture was diluted to 1 ml with nuclease S1 digestion buffer (30 mM sodium acetate, pH 4.5, 1 mM zinc sulfate, 5% glycerol), and 100 units of S1 nuclease was added. After incubation at 45°C for 60 min, the reaction was terminated by the addition of 5% trichloroacetic acid and the acid-insoluble radioactivity determined.

DNA Binding Experiments—A simple procedure was devised to analyze the binding of both helicase III and *rep* protein to DNA. The enzyme to be analyzed was incubated with the DNA for 4 min at 37°C, and then the sample was layered onto 0.12 ml of glycerol solution (30%) containing 50 mM Tris-HCl, pH 8.0, 40 mM NaCl, 4

Enzymes capable of catalyzing DNA strand separation in an ATP-dependent reaction have been isolated from uninfected (1-8) and bacteriophage-infected (9-12) cells of *Escherichia coli*. These enzymes can be classified as acting either catalytically or stoichiometrically, depending upon the amount of enzyme required to promote a given amount of strand separation. The *rep* protein (13, 14), *recBC* enzyme (15), T7 gene 4 protein, and T4 genes 44/62 and gene 45 proteins (10, 11) all act catalytically, whereas helicases I and II most probably act stoichiometrically (16, 17). Our previous

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mM dithiothreitol, and centrifuged for 15 min at 22°C in a Beckman Airfuge. Samples were then fractionated by puncturing the bottom of the nitrocellulose tubes and collecting 0.008-ml fractions through a 25-gauge needle. The samples were then assayed for DNA-dependent ATPase activity.

RESULTS

Effect of DNA Binding Proteins on the DNA-dependent ATPase Activity—We have shown previously that both the *rep* protein and the helicase III-mediated DNA unwinding require ATP and *E. coli* DNA binding protein (14, 19). The *E. coli* DNA binding protein, however, can completely abolish the single-stranded DNA-dependent ATPase associated with the *rep* protein. We postulated (21) that both *rep* and DNA binding protein competed for the same or overlapping sites on single-stranded DNA, and thus, when binding protein was bound first, *rep* protein could not bind. During unwinding, the DNA binding protein, not having a strong affinity for double-stranded DNA, could participate in the unwinding reaction by binding to single-stranded DNA “behind” the advancing *rep* protein. Accordingly, we wanted to see whether helicase III behaved similarly to *rep* protein with regard to the effect of DNA binding protein on the ATPase activity and also whether the *E. coli* DNA binding protein could be replaced by the bacteriophage T4-induced binding protein, the helix-destabilizing protein.

The results in Fig. 1 demonstrate that the single-stranded DNA-dependent ATPase activity of both *rep* protein and helicase III could be abolished by addition of the *E. coli* DNA binding protein. The amount of binding protein required to give essentially complete inhibition is that amount required to just saturate the added single-stranded DNA. It is also shown that the T4 helix-destabilizing protein can abolish the ATPase activity; however, approximately 5-fold more than a stoichiometric amount is required to inhibit *rep* protein-catalyzed ATPase than helicase III-catalyzed activity. This dif-

TABLE I

Requirements for duplex DNA unwinding

Standard reaction conditions were employed. Each reaction contained 125 units of Fraction V enzyme.

Condition	Duplex unwound %
Complete	42
– <i>E. coli</i> DNA binding protein	10
–ATP	<5
– <i>E. coli</i> DNA binding protein + T4 helix-destabilizing protein	20
–Mg ²⁺	<5

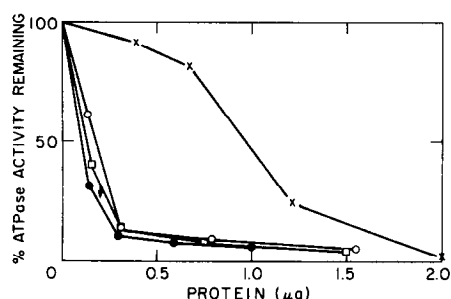


FIG. 1. DNA binding protein inhibition of DNA-dependent ATPase activity. Reactions contained standard assay buffer, ss fd DNA (0.024 μg), helicase III (15 units), and DNA binding protein as shown. Initial reaction rates were measured, and 100% activity represents the hydrolysis of 7.5 nmol of ATP. ●—●, helicase III + *E. coli* DNA binding protein; ○—○, helicase III + T4 DNA binding protein; ×—×, *rep* + T4 DNA binding protein; □—□, *rep* + *E. coli* DNA binding protein.

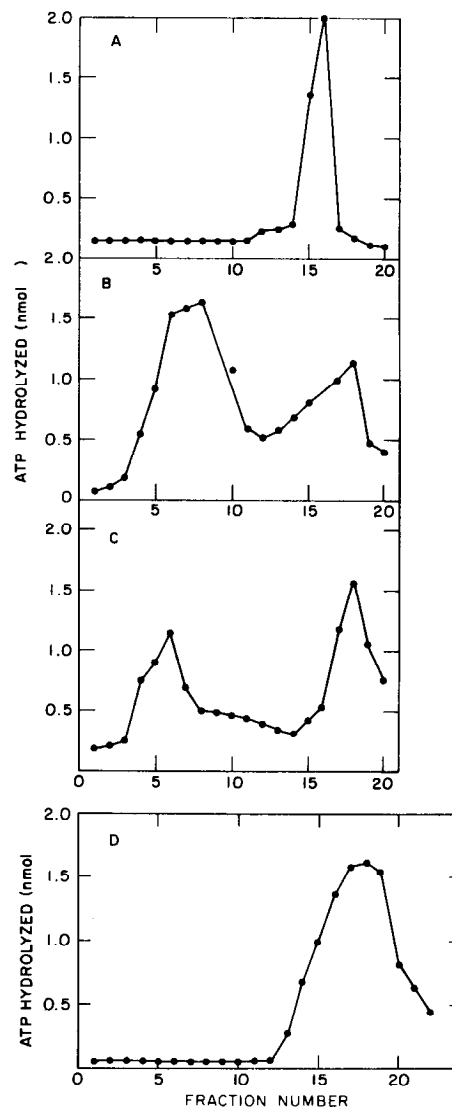


FIG. 2. Analysis of binding of *rep* protein to DNA. The binding experiments were performed as described under “Experimental Procedures.” A, *rep* protein (50 units) + DNA binding protein (0.3 μg); B, *rep* protein + ss fd DNA (0.024 μg); C, *rep* protein incubated with DNA, followed by incubation with DNA binding protein; D, ss fd DNA made to form complex first with DNA binding protein and then incubated with *rep* protein. Sedimentation is from right to left.

ference may reflect the fact that *rep* protein has a higher affinity for single-stranded DNA than helicase III does (see below).

We next examined directly the binding of *rep* protein and helicase III to single-stranded DNA and also studied the effect of the *E. coli* DNA binding protein in that reaction. Binding of *rep* protein and helicase III to DNA was studied by incubating the two macromolecules together and then subjecting the mixture to centrifugation. The presence of protein was detected by its ATPase activity. Under the conditions employed, free protein sedimented only slightly and DNA sedimented rapidly. The details are given under “Experimental Procedures.” Figs. 2 and 3 represent the results obtained with the *rep* protein and helicase III, respectively.

Panels A in Figs. 2 and 3 show the sedimentation behavior of ATPase activity in the presence of DNA binding protein but in the absence of DNA. **Panels B** in Figs. 2 and 3 show the sedimentation behavior in the presence of single-stranded DNA but in the absence of DNA binding protein. If *rep* protein or helicase III are mixed with DNA and then DNA binding protein is added, the results shown in **Panels C** of

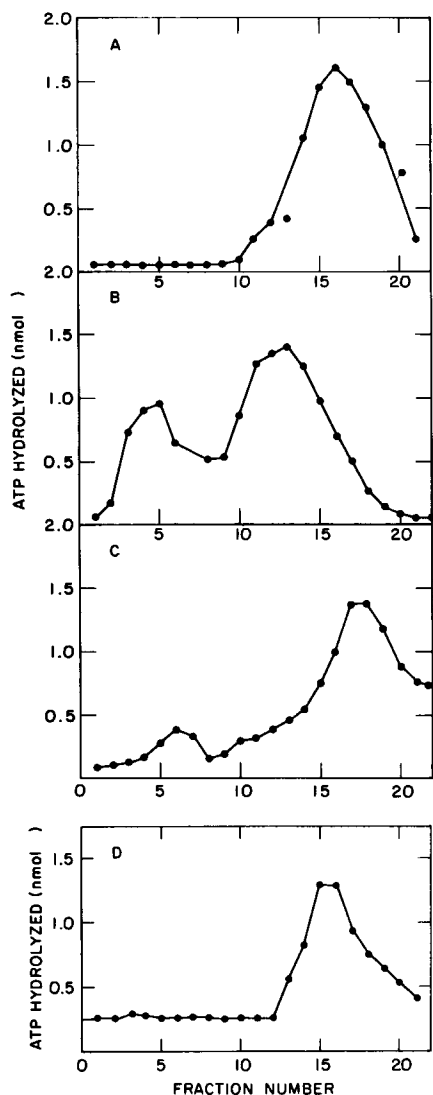


FIG. 3. Analysis of binding of helicase III to DNA. The binding experiments were performed as described under "Experimental Procedures." A, helicase III (50 units) + DNA binding protein (0.2 μ g); B, helicase III + ss fd DNA (0.024 μ g); C, helicase III incubated with DNA, then with DNA binding protein; D, ss fd DNA first made to form complex with DNA binding protein and then incubated with helicase III. Sedimentation is from right to left.

Figs. 2 and 3 are obtained. We conclude from these results that both proteins bind to single-stranded DNA and that DNA binding protein added in amounts stoichiometric with DNA does not displace either *rep* protein or helicase III from DNA. In contrast, if DNA binding protein is added to DNA first and then the *rep* protein or helicase III is added, no binding is observed (Panels D, Figs. 2 and 3). Thus it appears that the inhibition of ATPase activity caused by DNA binding protein is a result of competition for binding to DNA. This result is in keeping with our previous conclusions concerning the interaction of *rep* protein with DNA (14).

It also appears from examination of the results in Figs. 2 and 3 that helicase III has a higher rate of dissociation from single-stranded DNA than *rep* protein does. This rate has not been quantitated, but as seen in Fig. 3, Panel C, only a small amount of helicase III remains on the DNA after sedimentation compared to that seen with *rep* protein. The number of units of ATPase added in each case is equivalent.

We have also examined the effect of inclusion of ATP in the binding assays. Those represented here were performed

in the absence of ATP. We have not observed any significant differences in the presence of ATP.

DNA Unwinding by Helicase III—To test the properties of helicase III in its ability to catalyze unwinding of duplex DNA, partially duplex, circular DNA substrates were constructed by annealing 32 P-labeled ϕ X174 DNA restriction fragments to viral circles (see under "Experimental Procedures"). DNA unwinding was measured as the generation of nuclease S1-sensitive 32 P-labeled DNA following incubation of the partial duplex DNA with helicase III. A product analysis of the unwinding reaction by sucrose gradient sedimentation is described in the preceding paper (19). As previously shown for *rep* protein-dependent unwinding of DNA, *E. coli* DNA binding protein is required (see Table 1). Fig. 4 demonstrates the effect of DNA binding protein on the reaction catalyzed by helicase III. When 120 units of enzyme (Fig. 4, open circles) is used, binding protein stimulates the extent of unwinding 4-fold. The amount of binding protein showing maximal stimulation is that amount sufficient to complex 20% of the single-stranded DNA added. Higher concentrations of binding protein give inhibition. Inhibition at high binding protein concentrations was also seen with *rep* protein (14) and presumably is due to the prevention of binding of helicase III to the substrate (see Figs. 2 and 3). Unlike that seen with the *rep* protein, increasing the amount of helicase III results in a binding protein-independent reaction (Fig. 4, filled circles). We have reported (19) that DNA binding protein does contaminate the helicase III preparation and may account for the latter result. The extent of binding protein-independent unwinding seen cannot be stimulated by the further addition of DNA binding protein or by the addition of higher concentrations of helicase III (see Fig. 5). The basis of this phenomenon has not been explored further.

Substitution of the Bacteriophage T4 Helix-destabilizing Protein for the *E. coli* DNA Binding Protein—We have previously shown that *E. coli* DNA binding protein is capable of forming specific protein complexes with a variety of enzymes involved in DNA metabolism. Furthermore, the inter-

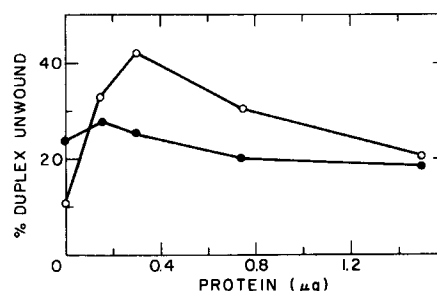


FIG. 4. Effect of *E. coli* DNA binding protein on the extent of unwinding. Experiments were performed at two enzyme concentrations: \circ — \circ , 125 units; \bullet — \bullet , 250 units.

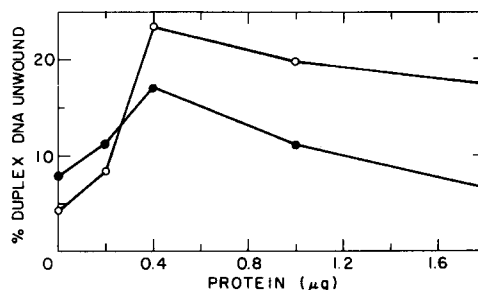


FIG. 5. A comparison of the effect of T4 DNA binding protein on the extent of unwinding of *rep* and helicase III. Reactions contained 125 ATPase units and DNA binding protein as shown. \circ — \circ , *rep* protein; \bullet — \bullet , helicase III.

actions appear to be specific in that only the *E. coli* DNA binding protein and not the T4 protein can stimulate DNA polymerases from *E. coli*. In addition, the *E. coli* protein stimulates DNA polymerase II activity but inhibits DNA polymerase I activity (20). In order to gain further insight into the role of DNA binding protein in the unwinding reaction, we attempted to substitute the T4 protein for the *E. coli* protein. As shown in Fig. 5 and Table 1, the T4 protein does in fact satisfy the binding protein requirement in this reaction both with *rep* protein-catalyzed unwinding (Fig. 5, *open circles*) and helicase III-catalyzed unwinding (Fig. 6, *filled circles*). These results suggest that the role of the binding protein at least on these artificial substrates does not require the interaction of enzyme and binding protein. As shown below, this result is not obtained if more natural substrates are employed.

Directional Specificity of Duplex Unwinding—We have shown previously (14) that *rep* protein will catalyze unwinding of partially duplex DNA provided that a single-stranded leader is present on the molecule. The leader must have a free 3' end. Flush-ended duplexes, nicked duplexes, or those with a single-stranded tail having a free 5' terminus are not unwound. Since *rep* protein does not bind to duplex DNA, as judged by ATPase activity, we assume that the protein binds to the single-stranded tail and moves in a 3' to 5' direction along the single strand until the duplex portion is reached, at which point unwinding is catalyzed. Therefore, by convention we now say that *rep* moves in a 3' to 5' direction.

The results of testing directional specificity for helicase III and *rep* protein are shown in Fig. 6, A and B, respectively. In keeping with our previous finding, *rep* protein catalyzed unwinding in a 3' to 5' direction. In contrast, helicase III unwinds DNA by moving in the opposite direction, namely 5' to 3'. It should be noted that the directional specificity of helicase III is also maintained in the DNA binding protein-independent reaction. Thus we conclude that *rep* protein and helicase III move in opposite directions along single-stranded DNA and that, although DNA binding protein greatly stimulates the

TABLE II
Comparison of *rep* and helicase III substrate specificities for unwinding

All unwinding conditions were as previously described (14).

Condition	Per cent of duplex unwound			
	<i>rep</i> protein		helicase protein	
	ϕ XRF I-cisA	Synthetic	ϕ XRF I-cisA	Synthetic
–DNA binding protein	<2	5	<1	10
+T4 helix-destabilizing protein	7	23	<1	18
+ <i>E. coli</i> DNA binding protein	35	20	<1	40

reaction, it appears not to influence the direction of movement.

Bacteriophage ϕ X174RF I Unwinding—The *rep* protein is able to catalyze the unwinding of ϕ X174RF I DNA provided that the DNA has been cut by the *cisA* protein first and ATP and DNA binding protein are added. It was of interest to see first whether the T4 helix-destabilizing protein could substitute for the *E. coli* protein in this reaction, and second whether the *rep* protein could be replaced by helicase III. The data in Table II summarize the results of this study. Although the T4 helix-destabilizing protein can substitute for the *E. coli* DNA binding protein in the unwinding of the synthetic duplex molecules, it cannot replace the *E. coli* protein for the unwinding of the *cisA*-cut ϕ X174RF I DNA. Furthermore, helicase III cannot replace the *rep* protein for the ϕ X174 DNA unwinding reaction. Thus the specificity that is seen *in vivo* is preserved *in vitro*.

DISCUSSION

Our results show that the novel DNA-dependent ATPase activity described here and designated helicase III is very similar to the *rep* protein in its DNA unwinding properties and interaction with *E. coli* DNA binding protein. The cofactor activity of single-stranded DNA for the DNA-dependent ATPase activities of both *rep* and helicase III is lost when *E. coli* DNA binding protein coats the DNA. We have shown directly that this inhibition is due to inability of either ATPase enzyme to bind to cofactor in a complex with DNA binding protein. When the enzymes are bound to DNA before the addition of DNA binding protein, enzyme-DNA complex can be recovered after a brief incubation, suggesting that DNA binding protein does not displace bound enzyme from the substrate. However, we have not measured directly the rates of dissociation of the enzyme from the DNA in the presence and absence of DNA binding protein.

In common with the *rep* protein (14), helicase III is also capable of unwinding partially duplex DNA, but not duplex DNA containing only a nick. In contrast to *rep* protein, significant unwinding was observed in the absence of added *E. coli* DNA binding protein, although the latter did stimulate the extent of reaction at limiting enzyme concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reported in the preceding paper (19), suggests that DNA binding protein is a minor contaminant protein in our preparations and may account for the apparent DNA binding protein independence. Alternatively, the enzyme may be capable of strand separation by itself provided enough molecules are present at the site of duplex DNA invasion to separate the strand completely before renaturation occurs. If the latter were true, it may explain why binding protein does not increase the extent of reaction at high helicase III concentrations.

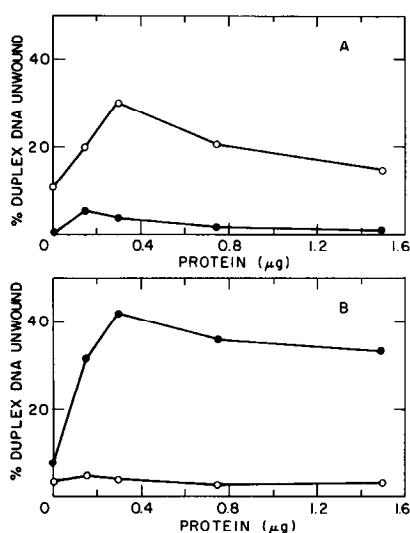


FIG. 6. Directional specificity of DNA duplex unwinding—a comparison of the direction of unwinding catalyzed by *rep* and helicase III. The substrates were as described under "Experimental Procedures" in the accompanying paper (19). A, helicase III, unwinding partial duplex in (○—○) 5' to 3' and (●—●) 3' to 5'; B, *rep* protein (○—○) 5' to 3' and (●—●) 3' to 5'. (The limited amount of unwinding seen with the 3' to 5' substrate with helicase III and vice versa with *rep* is due to a small fragment with opposite polarity present in the substrate (14).)

An insight into the role of DNA binding protein on the partial duplex unwinding reaction may be gained from the results of our experiments in which *E. coli* DNA binding protein was replaced by T4 helix-destabilizing protein. For both *rep* protein and helicase III, T4 helix-destabilizing protein could replace *E. coli* DNA binding protein function, suggesting that there is probably no specific helicase-binding protein interaction and that DNA binding protein plays a passive role, preventing renaturation during strand separation. Although T4 helix-destabilizing protein can replace *E. coli* DNA binding protein in the partial duplex unwinding reaction catalyzed by *rep* protein, it could not do so in the unwinding of ϕ XRF I. This finding suggests that the *E. coli* DNA binding protein may play a role in the initiation of unwinding on this substrate, or that it interacts with *rep* protein or *cisA* protein directly in the unwinding process. All of these results, including the finding of directional specificity (see below), support our suggested model for the mechanism of unwinding (14).

In common with other DNA unwinding enzymes (12, 14, 16, 17), helicase III shows directional specificity with regard to DNA unwinding. Helicase III moves along single-stranded DNA in a 5' to 3' direction and invades duplex DNA. This is the same directional specificity as has been reported for helicases I and II (16, 17). The *rep* protein moves along DNA with the opposite polarity.

Although both proteins may move along the DNA in opposite directions by attaching to opposite strands of the helix at a replication fork, they could both move with the replication fork, since DNA strands are antiparallel. Previous work by others (18) has shown that, although the *rep* mutation is not lethal to the cell, the rate of replication fork movement is slower in *rep*⁻ mutants. We propose that both these proteins could be involved in unwinding duplex DNA in the cell, and that loss of either one independently would not be a lethal event, although the rate of fork movement may be reduced. To test this hypothesis, we are searching for mutants that are conditionally lethal in *rep*-deficient backgrounds but not in *rep*-proficient backgrounds. By analogy with *rep*⁻ mutants, if our proposed scheme is correct, such a mutation in a *rep*⁺

strain would lead to slower rates of replication fork movement.

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