

The Replicative Helicases of Bacteria, Archaea, and Eukarya Can Unwind RNA-DNA Hybrid Substrates^{*[5]}

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Replicative helicases are hexameric enzymes that unwind DNA during chromosomal replication. They use energy from nucleoside triphosphate hydrolysis to translocate along one strand of the duplex DNA and displace the complementary strand. Here, the ability of a replicative helicase from each of the three domains, bacteria, archaea, and eukarya, to unwind RNA-containing substrate was determined. It is shown that all three helicases can unwind DNA-RNA hybrids while translocating along the single-stranded DNA. No unwinding could be observed when the helicases were provided with a single-stranded RNA overhang. Using DNA, RNA, and DNA-RNA chimeric oligonucleotides it was found that whereas the enzymes can bind both DNA and RNA, they could translocate only along DNA and only DNA stimulates the ATPase activity of the enzymes. Recent observations suggest that helicases may interact with enzymes participating in RNA metabolism and that RNA-DNA hybrids may be present on the chromosomes. Thus, the results presented here may suggest a new role for the replicative helicases during chromosomal replication or in other cellular processes.

Biochemical studies with the replicative helicases of bacteria, archaea, and eukarya suggest that these enzymes form ring-shaped structures that encircle and translocate along DNA while utilizing the energy derived from NTP hydrolysis to separate duplex DNA at the front of the replication fork (1). The bacterial DnaB helicase is a homohexamer that binds and translocates along single-stranded (ss)² and double-stranded (ds) DNA and possesses a 5'→3' helicase activity and DNA-dependent ATPase activity (2). The eukaryotic minichromosome maintenance (MCM) complex is a family of six related polypeptides (Mcm2–7), each of which is essential for cell viability. Biochemical studies have shown that a dimeric complex of the Mcm4,6,7 heterotrimer contains 3'→5' DNA helicase

activity, ssDNA binding, and DNA-dependent ATPase activity and is capable of translocating along ss and dsDNA. *In vitro*, the Mcm2 and Mcm3,5 complexes were shown to inhibit helicase activity (3, 4). Although it has not yet been shown, the MCM complex is thought to function as the eukaryotic replicative helicase. In most archaeal species studied, a single MCM homologue has been identified. The structure of the protein is not yet clear; hexamers, heptamers, dodecamers, and filaments have been reported (5). Biochemical studies revealed that the archaeal enzyme possesses an ATP-dependent 3'→5' helicase activity, DNA-dependent ATPase activity, and can bind and translocate along ss and dsDNA (5, 6).

The eukaryotic MCM complex was shown to interact with RNA polymerase (7, 8) and was found in a complex with Yph1 (9), a protein needed for 60 S ribosomal biogenesis that may also participate in polysome translation. Thus it is possible that some replicative helicases participate in cellular processes involving RNA in addition to their role in chromosomal DNA replication. To date, it has not been established whether the MCM complex is capable of translocating and/or unwinding RNA or DNA-RNA hybrids.

A study was therefore initiated to determine the ability of replicative helicases from the three domains, bacteria, archaea, and eukarya, to unwind RNA-containing substrates. It is shown here that the *Escherichia coli* DnaB protein (ecDnaB), the *Methanothermobacter thermautotrophicus* MCM protein (mtMCM), and the *Schizosaccharomyces pombe* Mcm4,6,7 complex (spMCM) cannot unwind duplex RNA substrates. All can, however, unwind a DNA-RNA hybrid substrate when translocating along the ssDNA strand, but not when only overhanging ssRNA was provided. It was also found that although all three enzymes can bind RNA, RNA cannot stimulate the ATPase activity of the enzymes.

EXPERIMENTAL PROCEDURES

Materials

ATP and [γ -³²P]ATP were obtained from GE Healthcare, and oligonucleotides and biotinylated oligonucleotides were synthesized by the CARB DNA synthesis facility. Biotin was obtained from Sigma, streptavidin from Rockland Immunochemicals, and nitroavidin (CaptAvidinTM) from Molecular Probes. Biotin-dT and 2-*o*-methyl NTPs were obtained from Glen Research. mtMCM was purified as previously described (10), spMCM was kindly provided by Dr. Jerard Hurwitz (Memorial Sloan-Kettering Cancer Center) and Dr. Joon-Kyu Lee (Seoul National University), and ecDnaB was kindly provided by Dr. Mike O'Donnell (The Rockefeller University).

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2 and Table S1.

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² The abbreviations used are: ss, single-stranded; ds, double-stranded; ec, *E. coli*; MCM, minichromosome maintenance; mt, *M. thermautotrophicus*; sp, *S. pombe*.

Methods

Preparation of Substrates for Helicase Assays—Oligonucleotides used for the preparation of the different helicase substrates are listed in supplemental Table S1. The helicase substrates were made as follows. Five pmol of DNA or RNA oligonucleotides (as indicated in the figure legends) were labeled at the 5'-end using 16.7 pmol [γ - 32 P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase. Labeling reactions were stopped by adding EDTA to a final concentration of 25 mM. The labeled oligonucleotide was hybridized to the complementary oligonucleotide(s) at a 1:2 molar ratio in a buffer containing 40 mM Hepes-NaOH, pH 7.5, and 50 mM NaCl by heating to 100 °C for 3 min followed by slow cooling to 25 °C. After hybridization, 5 \times DNA loading buffer (0.1% xylene cyanol, 0.1% bromophenol blue, and 50% glycerol) was added to a final concentration of 1 \times , and the mixture was electrophoresed through an 8% native polyacrylamide gel for 1 h at 100 V in 0.5 \times TBE (45 mM Tris, 4.5 mM boric acid, 0.5 mM EDTA) to remove unincorporated [γ - 32 P]ATP and free oligonucleotides. The substrates were located by autoradiography and the products were excised from the gel, which was sliced into small pieces and incubated at 37 °C for 16 h in 3 volumes of an elution buffer containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0. After centrifugation, the supernatants were collected and the substrates were ethanol precipitated and resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Due to low recovery of duplex RNA substrates, 10-fold more RNA oligonucleotide (50 pmol) was 5'-end labeled with the same amount of [γ - 32 P]ATP (16.7 pmol, 3,000 mCi/mmol) and T4 polynucleotide kinase. The specific activity of the DNA substrates was \sim 3,000 cpm/fmol and that of duplex RNA \sim 500 cpm/fmol.

DNA Helicase Assay—DNA helicase activity of the ecDnaB was measured in reaction mixtures (15 μ l) containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 5 mM ATP, 10 fmol 32 P-labeled DNA substrate, and ecDnaB protein as indicated in the figure legends. Mixtures were incubated at 37 °C for the time indicated in the legends for Figs. 1, 3, 6, and supplemental Figs. S1 and S2.

DNA helicase activity of the mtMCM was measured in reaction mixtures (15 μ l) containing 20 mM Tris-HCl, pH 8.5, 10 mM MgCl₂, 2 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 5 mM ATP, 10 fmol 32 P-labeled DNA substrate, and mtMCM proteins as indicated in the figure legends. Mixtures were incubated at 60 °C for the time indicated in the legends for Figs. 1, 3, 6, and supplemental Figs. S1 and S2.

DNA helicase activity of the spMCM complex was measured in reaction mixtures (15 μ l) containing 25 mM Hepes-NaOH, pH 7.5, 25 mM potassium acetate, 10 mM magnesium acetate, 5 mM ATP, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 10 fmol 32 P-labeled DNA substrate, and spMCM complex as indicated in the figure legends. Mixtures were incubated at 30 °C for the time indicated in the legends for Figs. 1, 3, 6, and supplemental Figs. S1 and S2.

Reactions were stopped by adding 5 μ l of loading buffer containing 2% SDS, 100 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, and 50% glycerol and chilling on ice. Aliquots were

loaded onto an 8% native polyacrylamide gel in 0.5 \times TBE and electrophoresed for 1.5 h at 200 V at 4 °C. Gels were visualized and quantitated by phosphorimaging. All helicase experiments were repeated at least three times, and their averages with standard deviations are shown in the figures together with representative gels.

Filter Binding Assay—Oligonucleotides for DNA and RNA filter binding assays were prepared by labeling the oligonucleotide using [γ - 32 P]ATP and T4 polynucleotide kinase. Unincorporated [γ - 32 P]ATP was removed from the single-stranded substrate using Sephadex G-50 column chromatography.

Nitrocellulose filter binding assays were performed with 50 fmol 32 P-labeled substrate. The binding reactions were performed at 60 °C (mtMCM), 30 °C (spMCM) or 37 °C (ecDnaB) for 10 min in 20 μ l of reaction buffer containing 20 mM Hepes-NaOH, pH 7.5, 10 mM MgCl₂, 2 mM dithiothreitol, and 100 μ g/ml bovine serum albumin. After incubation, the mixture was filtered through an alkaline-washed nitrocellulose filter (Millipore, HA 0.45 μ m) (11), which was subsequently washed with 20 mM Hepes-NaOH, pH 7.5. The radioactivity adsorbed to the filter was measured by liquid scintillation counting. All DNA binding experiments were repeated three times, and their averages with standard deviations are shown in Fig. 4.

Streptavidin/Nitroavidin Displacement Assay—Biotinylated oligonucleotides were 5'-end labeled using [γ - 32 P]ATP and T4 polynucleotide kinase and purified as described above for the preparation of substrates for filter binding assay. Ten fmol of oligonucleotides (as indicated in the figure legends) were incubated with 750 fmol streptavidin for the experiments with the mtMCM or with 1.5 pmol nitroavidin for the experiments with spMCM and ecDnaB in the respective helicase reaction mixtures (15 μ l) at 30 °C for 10 min. Next, the helicase was added together with 10 pmol free biotin for each reaction (to trap and sequester streptavidin or nitroavidin when released from the oligonucleotides by the helicase). After incubation for 1 h at 55 °C (mtMCM), 30 °C (spMCM), or 37 °C (ecDnaB), 5 μ l of 5 \times loading buffer (100 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, and 50% glycerol) was added to each sample, followed by electrophoretic analysis on a native 8% polyacrylamide gel in 0.5 \times TBE. The gels were analyzed using phosphorimaging. All experiments were repeated three times with almost identical results. Representative gels are shown in Fig. 2.

ATPase Assay—ATPase activity was measured in reaction mixtures (15 μ l) containing 25 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 1.5 nmol ATP containing 0.495 pmol [γ - 32 P]ATP (3000 Ci/mmol; Amersham Biosciences), and 90 fmol mtMCM (as hexamer) or 270 fmol spMCM and ecDnaB (as hexamer) in the presence or absence of 2 pmol 25-base DNA or RNA oligonucleotide as indicated in the legend of Fig. 5. After incubation at 60 °C (mtMCM), 30 °C (spMCM), or 37 °C (ecDnaB) for 60 min, a 1- μ l aliquot was spotted onto a polyethyleneimine cellulose thin layer plate, and ATP and P_i were separated by chromatography in 1 M formic acid and 0.5 M LiCl. The extent of ATP hydrolysis was quantitated by phosphorimager analysis.

RESULTS

The Replicative Helicases of Bacteria, Archaea, and Eukarya Can Unwind DNA-RNA Hybrid—It has not yet been determined whether the archaeal or eukaryal MCM proteins are capable of unwinding substrates containing RNA, and only limited studies have been performed with the bacterial DnaB protein (12). Thus, the ability of a replicative helicase from bacteria, archaea, and eukarya to unwind RNA-containing substrate was determined.

It was shown previously that the spMCM complex is unable to displace a DNA substrate with a duplex region of 20 bp or longer when provided with only a 3'-ssDNA overhang (Ref. 13 and supplemental Fig. S1). To unwind that substrate, the helicase required a forked DNA structure containing both 3'- and 5'-overhanging ssDNA (13, 14). Similar observations have been made with the bacterial DnaB protein (supplemental Fig. S1). Therefore, all substrates used in the first set of experiments (Fig. 1) contained duplex regions of 25 bp in length and forked structures (containing both 3'- and 5'-overhang single-stranded regions) as shown schematically at the top of each panel in Fig. 1.

First, as a control, the ability of the enzymes to unwind duplex DNA was determined. All three helicases can unwind such substrates with comparable efficiencies to those previously reported for the enzymes (Fig. 1, A–C, lanes 3–5 (I), see also panels D–F). Next, the ability of the helicase to unwind duplex RNA substrates was determined. None of the enzymes, mtMCM, spMCM, or ecDnaB, could unwind these substrates (Fig. 1, A–C, lanes 18–20 (IV), see also panels D–F).

Although the enzymes cannot unwind duplex RNA, they may have the ability to unwind substrates containing RNA-DNA hybrids. There are two types of such hybrid substrates. One contains 3'-overhanging DNA and 5'-overhanging RNA, whereas the other has the reverse, 3'-overhanging RNA and 5'-overhanging DNA. The ability of the enzyme to unwind both types of substrates was evaluated.

As shown in Fig. 1, A–C, all three helicases are capable of unwinding hybrid DNA-RNA substrates when translocating along the DNA strand (Fig. 1, A–C, lanes 8–10 (II), see also panels D and E; recall that the eukaryotic and archaeal MCM have a 3'→5' polarity on ssDNA whereas DnaB moves in the 5'→3' direction). No unwinding could be observed, however, when the *M. thermautotrophicus* and *S. pombe* MCM proteins were provided with a 3'-RNA overhang (Fig. 1, A and B, lanes 13–15 (III), see also panels D and E) or when the ecDnaB was provided with 5'-RNA overhang (Fig. 1C, lanes 13–15 (III), see also panel F).

Interestingly, the *E. coli* enzyme unwinds hybrid RNA-DNA duplex more efficiently than duplex DNA (Fig. 1C, compare lanes 8–10 (II) with lanes 3–5 (I), see also panel F) whereas the MCM helicases are slightly inhibited (Fig. 1, A and B, compare lanes 8–10 (II) with lanes 3–5 (I), see also panels D and E). The reason for the difference is not clear. However, it may be due to the base composition of the substrates. It is well established that the stability of a DNA-RNA hybrid depends on which strand is the DNA and which is the RNA (e.g. Ref. 15). Thus, although the same oligonucleotide sequences were used to make the substrate for

DnaB and the MCM helicases, due to the different directionality of the DnaB and MCM (5'→3' and 3'→5', respectively), the DNA and RNA strands are “reversed” in the substrates used for ecDnaB in comparison with the two MCM proteins. It may be that the substrate used for the ecDnaB is less stable compared with that used for the MCM proteins.

The results presented in Fig. 1 demonstrate that although the helicases cannot unwind RNA substrates they can separate DNA-RNA hybrids if provided with a ssDNA region to move on. However, although the enzymes can unwind RNA-DNA hybrid substrates, the rate of unwinding a duplex DNA may be different from that for a RNA-DNA hybrid. Thus, the experiments described in Fig. 1 were repeated, varying the reaction time instead of helicase concentration (supplemental Fig. S2). Similar to the results of Fig. 1, both the eukaryal and archaeal MCM proteins are not as efficient at unwinding hybrid substrates in comparison with their activity on DNA substrates, but no clear difference in the rate of unwinding could be observed.

The Replicative Helicases Cannot Translocate along RNA—The results shown in Fig. 1 and supplemental Fig. S2 suggest that the helicases cannot move along RNA. To determine whether the enzyme can translocate along RNA, another, more direct approach was used.

It was previously shown that the mtMCM is capable of displacing streptavidin from biotinylated oligonucleotides while moving along DNA and that ATP is required for this activity (16). These experiments were performed using biotinylated oligonucleotides that were pre-bound by streptavidin. The mtMCM was incubated with the substrate in the presence of a large excess of biotin. The biotin in solution served as a trap to bind streptavidin upon its displacement from the DNA by the helicase.

A similar approach was used to determine whether the mtMCM is able to translocate along RNA. As shown in Fig. 2A, the enzyme can displace streptavidin from biotinylated DNA (lanes 3–6) and ATP is required for the activity (lane 7). No displacement could be observed when biotinylated RNA was used (lanes 10–13).

It was previously shown that the spMCM and ecDnaB could not displace streptavidin from biotinylated oligonucleotides (16, 17). Streptavidin binds biotin very tightly ($K_d \sim 10^{-15}$ M) (18), which may explain the inability of the eukaryal and bacterial helicases to remove it from biotinylated oligonucleotides. An avidin derivative, nitroavidin, binds biotin with much lower affinity ($K_d > 10^{-9}$ M) (19). Therefore, the ability of the ecDnaB and spMCM complex to displace nitroavidin from biotinylated oligonucleotide was analyzed in an experiment similar to that performed with streptavidin. It was found that both the spMCM (Fig. 2B, lanes 3–5) and ecDnaB (Fig. 2C, lanes 3–5) protein are capable of displacing nitroavidin from biotinylated DNA. Neither enzyme could do so if ATP was omitted from the reaction (Fig. 2, B and C, lanes 6), demonstrating that active movement by the helicase is required. In contrast, when biotinylated RNA was used instead of DNA, no nitroavidin displacement could be observed (Fig. 2, B and C, lanes 9–11), supporting the conclusion drawn from Fig. 1 and supplemental Fig. S2 that the enzymes cannot move along RNA.

Taken together, the data presented above demonstrate that the bacterial, archaeal, and eukaryal helicases cannot translo-

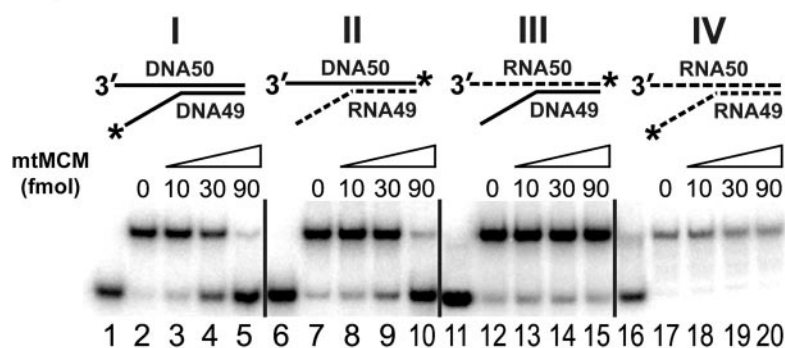
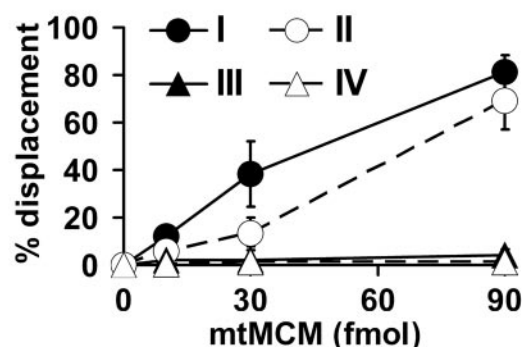
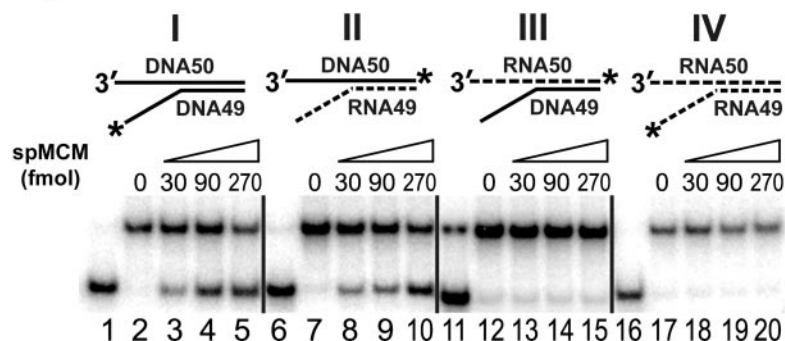
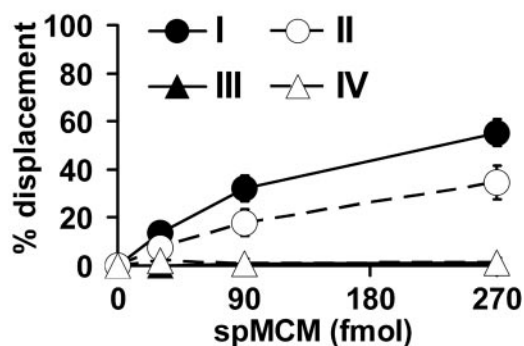
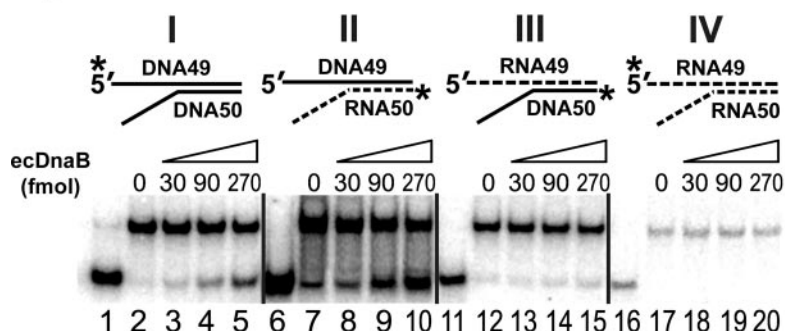
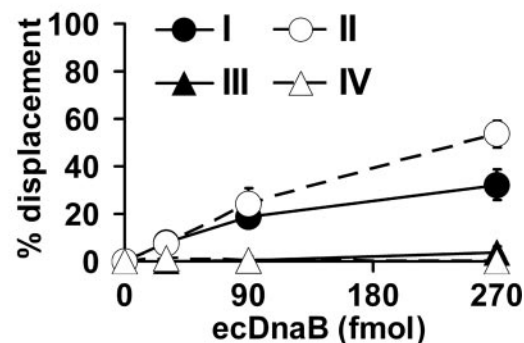
A) *M. thermautotrophicus***D)****B) *S. pombe*****E)****C) *E. coli*****F)**

FIGURE 1. mtMCM, ecDnaB, and spMCM can unwind DNA-RNA hybrid substrates. DNA helicase assays were performed as described under "Experimental Procedures" using 10 fmol substrate with increasing amounts of the mtMCM (A and D), spMCM (B and E), or ecDnaB (C and F) for 1 h at 60, 30, and 37 °C, respectively. Panels A–C are representative gels. 32 P-labeled strands are marked by an asterisk. DNA strands are represented by a solid line and RNA strands by a dashed line. Panel A, lanes 1, 6, 11, and 16, boiled substrate; lanes 2, 7, 12, and 17, substrate only; lanes 3, 8, 13, and 18, 10 fmol mtMCM as hexamer; lanes 4, 9, 14, and 19, 30 fmol mtMCM as hexamer; lanes 5, 10, 15, and 20, 90 fmol mtMCM as hexamer; lanes 4, 9, 14, and 19, 270 fmol mtMCM as hexamer. Panels B and C, lanes 1, 6, 11, and 16, boiled substrate; lanes 2, 7, 12, and 17, substrate only; lanes 3, 8, 13, and 18, 30 fmol spMCM (B) or ecDnaB (C) as hexamer; lanes 4, 9, 14, and 19, 90 fmol spMCM (B) or ecDnaB (C) as hexamer; lanes 5, 10, 15, and 20, 270 fmol spMCM (B) or ecDnaB (C) as hexamer. Panels D–F, summary of three independent experiments with standard deviation of the percent displacement by the mtMCM (D), spMCM (E), and ecDnaB (F). DNA/DNA substrate (I), closed circles; DNA/RNA substrate (II, translocation on the DNA strand), open circles; DNA/RNA substrate (III, translocation on the RNA strand), closed triangles; RNA/RNA substrate (IV), open triangles.

cate along RNA or unwind RNA duplexes. They all can, however, displace RNA from a RNA-DNA hybrid while moving along the DNA strand.

It is possible that the helicase cannot initiate translocation from RNA but could move along RNA if movement starts on DNA. Thus, the ability of the helicases to unwind substrates containing RNA-DNA chimeric oligonucleotides (schemati-

cally shown at the top of each panel of Fig. 3; DNA strands, solid line; RNA strands, dashed line) was determined. On these substrates, the helicase would either assemble on the DNA and then move to the RNA portion of the substrate or perform the reverse, assemble on the RNA and then move to the DNA. As shown in Fig. 3, each helicase is unable to unwind these chimeric substrates regardless of whether it first assembles on the

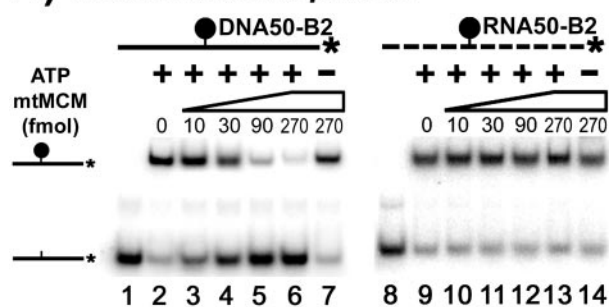
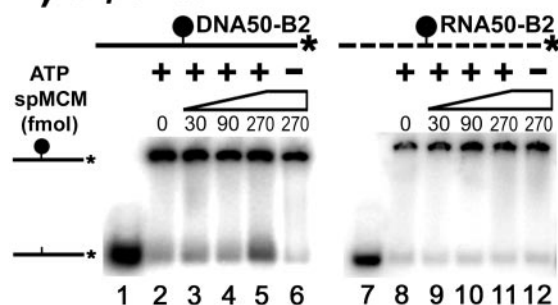
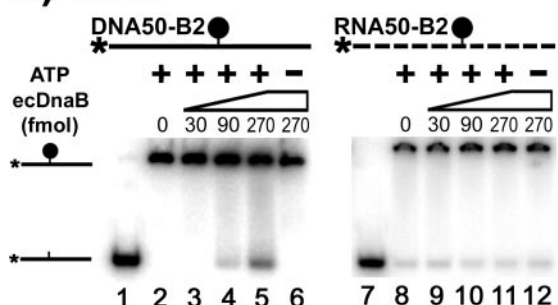
A) *M. thermautotrophicus***B) *S. pombe*****C) *E. coli***

FIGURE 2. mtMCM, ecDnaB, and spMCM can displace streptavidin or nitroavidin from biotinylated DNA but not RNA. Biotinylated ssDNA or ssRNA oligonucleotide was preincubated with a 75-fold molar excess of streptavidin (A) or a 150-fold molar excess of nitroavidin (B and C) at 30 °C for 10 min. Assays were performed as described under "Experimental Procedures" using 10 fmol substrate DNA or RNA with increasing amounts of mtMCM (A), spMCM (B), or ecDnaB (C) for 1 h at 55, 30, and 37 °C, respectively, in the presence of 10 pmol biotin. *Panel A*, lanes 1–7, DNA substrate; lanes 8–14, RNA substrate. Lanes 1 and 8, biotinylated oligonucleotide; lanes 2 and 9, biotinylated oligonucleotide bound to streptavidin; lanes 3 and 10, 10 fmol mtMCM as hexamer; lanes 4 and 11, 30 fmol mtMCM as hexamer; lanes 5 and 12, 90 fmol mtMCM as hexamer; lanes 6 and 13, 270 fmol mtMCM as hexamer; lanes 7 and 14, 270 fmol mtMCM as hexamer but without ATP. *Panels B and C*, lanes 1–6, DNA substrate; lanes 7–12, RNA substrate. Lanes 1 and 7, biotinylated oligonucleotide; lanes 2 and 8, biotinylated oligonucleotide bound to nitroavidin; lanes 3 and 9, 30 fmol spMCM (B) or ecDnaB (C) as hexamer; lanes 4 and 10, 90 fmol spMCM (B) or ecDnaB (C) as hexamer; lanes 5 and 11, 270 fmol spMCM (B) or ecDnaB (C) as hexamer; lanes 6 and 12, 270 fmol spMCM (B) or ecDnaB (C) as hexamer but without ATP. Streptavidin or nitroavidin is marked by a closed circle, and ³²P-labeled strands are marked by an asterisk.

RNA (Fig. 3, A–C, lanes 3–5) or DNA (lanes 8–10) portion of the substrate. These data demonstrate that assembly on DNA is not sufficient to promote RNA translocation and substrate unwinding by the helicases.

All Three Replicative Helicases Can Bind RNA—If the helicases cannot interact with RNA it would explain why the helicases can-

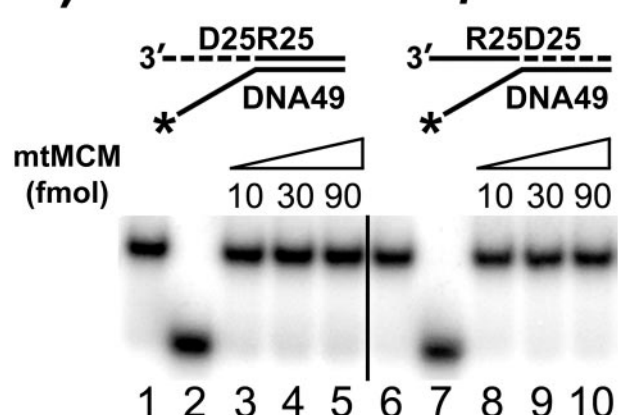
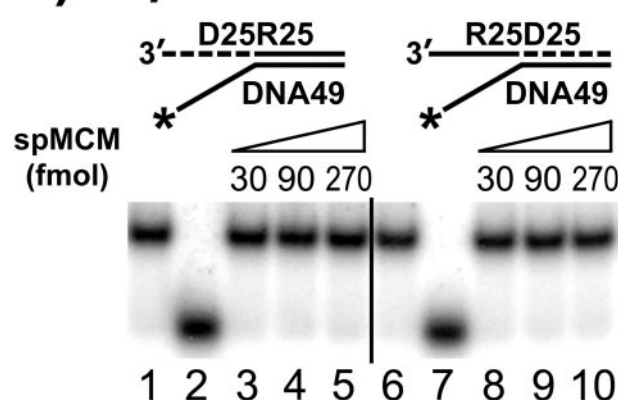
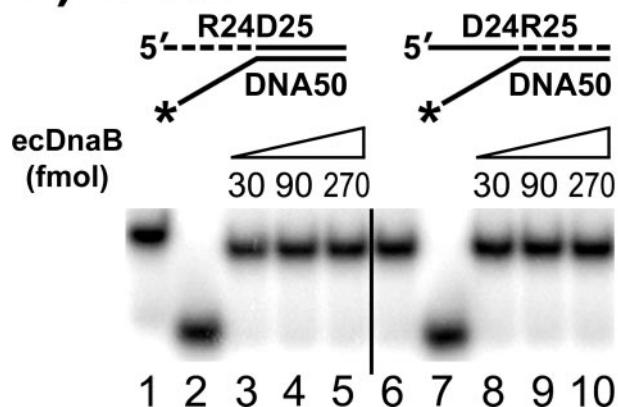
A) *M. thermautotrophicus***B) *S. pombe*****C) *E. coli***

FIGURE 3. mtMCM, ecDnaB, and spMCM are unable to unwind chimeric DNA-RNA substrates. DNA helicase assays were performed as described under "Experimental Procedures" using 10 fmol substrates with increasing amounts of the mtMCM (A), spMCM (B), or ecDnaB (C) for 1 h at 55, 30, and 37 °C, respectively. ³²P-labeled strands are marked by asterisks. DNA strands are represented by a solid line and RNA by a dashed line. *Panel A*, lanes 1 and 6, substrate only; lanes 2 and 7, boiled substrate; lanes 3 and 8, 10 fmol mtMCM as hexamer; lanes 4 and 9, 30 fmol mtMCM as hexamer; lanes 5 and 10, 90 fmol mtMCM as hexamer. *Panels B and C*, lanes 1 and 6, substrate only; lanes 2 and 7, boiled substrate; lanes 3 and 8, 30 fmol spMCM (B) or ecDnaB (C) as hexamer; lanes 4 and 9, 90 fmol spMCM (B) or ecDnaB (C) as hexamer; lanes 5 and 10, 270 fmol spMCM (B) or ecDnaB (C) as hexamer.

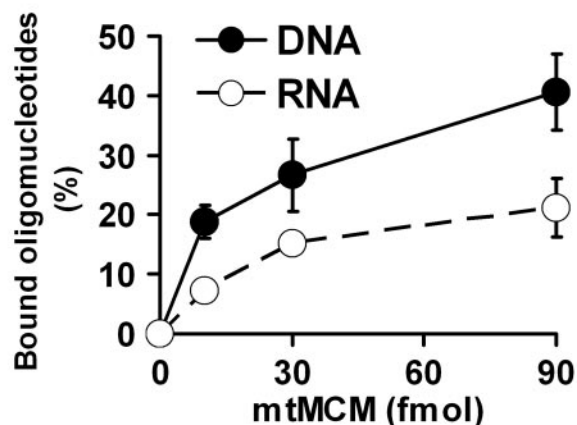
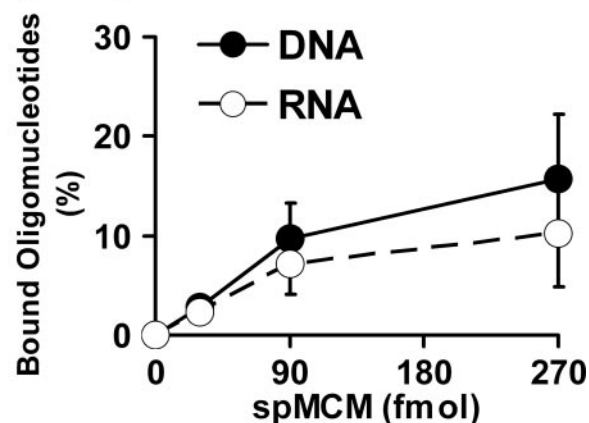
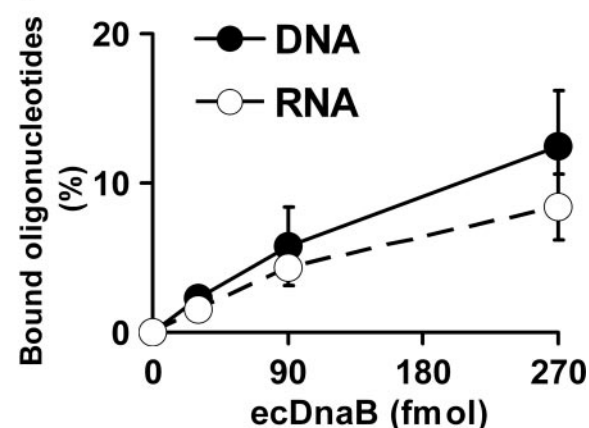
A) *M. thermautotrophicus***B) *S. pombe*****C) *E. coli***

FIGURE 4. mtMCM, ecDnaB, and spMCM bind DNA and RNA. DNA binding assays were performed as described under "Experimental Procedures" using 50 fmol 25-mer ssDNA (closed circles) or ssRNA (open circles) with increasing amounts of mtMCM (A), spMCM (B), or ecDnaB (C). A, reactions were performed in the presence of 30, 60, and 90 fmol mtMCM as hexamer. B and C, reactions were performed in the presence of 90, 180, and 270 fmol spMCM (B) or ecDnaB (C) as hexamer. Error bars indicate the standard deviation from three independent experiments.

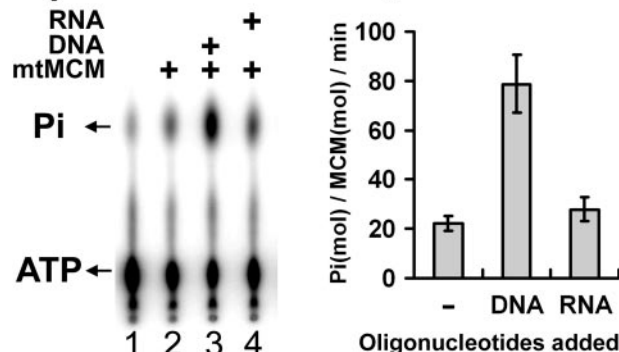
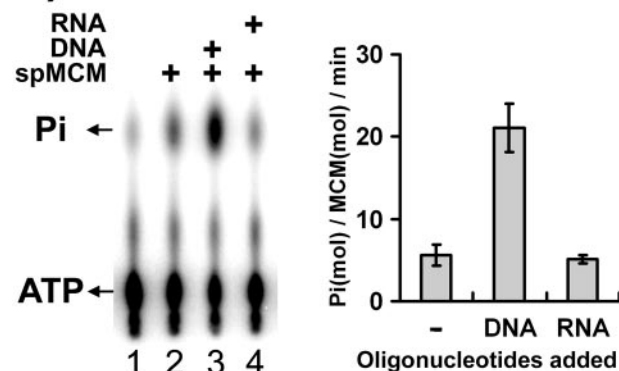
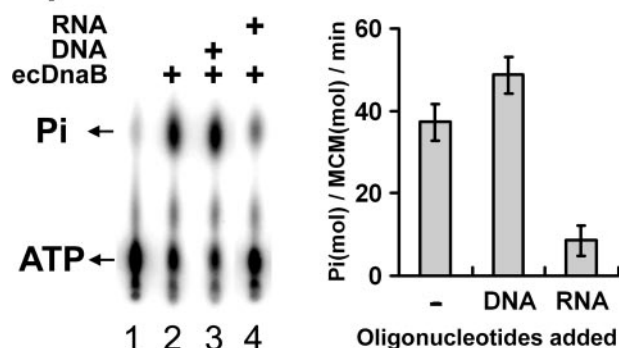
A) *M. thermautotrophicus***B) *S. pombe*****C) *E. coli***

FIGURE 5. DNA, but not RNA, stimulates the ATPase activity of mtMCM, ecDnaB, and spMCM. ATPase activity assays were performed as described under "Experimental Procedures" with the mtMCM (A), spMCM (B), or ecDnaB (C) protein in the absence or presence of 2 pmol 25-mer DNA or RNA for 1 h at 60, 30, and 37 °C, respectively. Representative results are shown together with a summary with standard deviation of three independent experiments.

not move along it. Therefore, the ability of the ecDnaB, mtMCM, and spMCM proteins to bind RNA was determined using 50-mer oligonucleotide in a filter binding assay. As shown in Fig. 4, all three enzymes bind RNA, though not as well as DNA. Nevertheless, the data suggest that lack of RNA binding is not the reason for the inability of the enzymes to move along it.

RNA Cannot Stimulate the ATPase Activity of the Replicative Helicases—The data presented in Fig. 4 demonstrate that the enzymes can bind RNA. However, to unwind the substrate the enzymes not only need to interact with the substrate but also to move along it. It is well established that the movement of

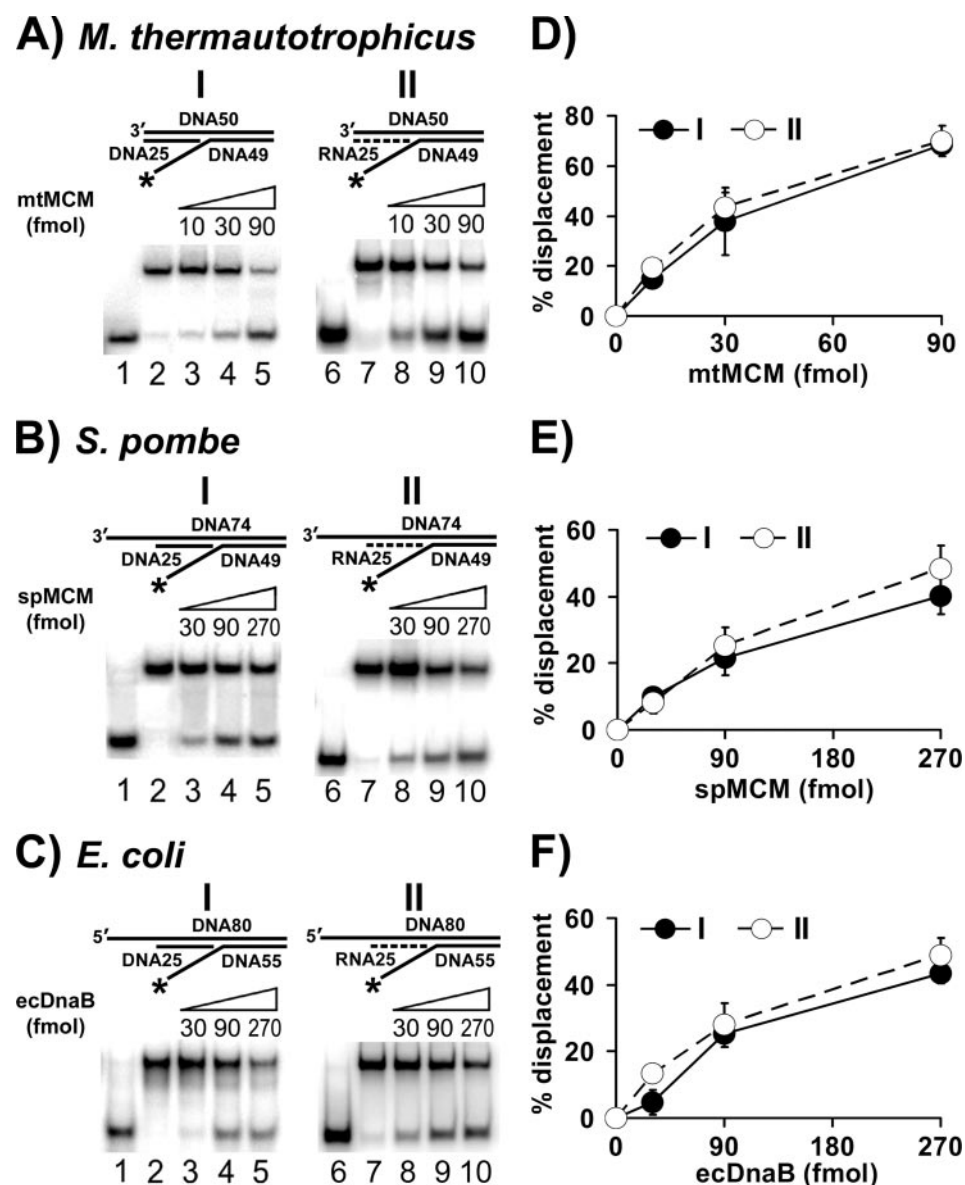


FIGURE 6. mtMCM, ecDnaB, and spMCM protein can translocate along a duplex DNA-RNA hybrid. Duplex translocation assays were performed as described under "Experimental Procedures" using 10 fmol substrate with increasing amounts of mtMCM (A and D), spMCM (B and E), or ecDnaB (C and F) protein for 1 h at 60, 30, and 37 °C, respectively. A–C, representative gels. 32 P-labeled strands are marked by an asterisk. DNA strands are marked by solid line and RNA strands by dashed line. Panel A, lanes 1 and 6, boiled substrate; lanes 2 and 7, substrate only; lanes 3 and 8, 10 fmol mtMCM as hexamer; lanes 4 and 9, 30 fmol mtMCM as hexamer; lanes 5 and 10, 90 fmol mtMCM as hexamer. Panels B and C, lanes 1 and 6, boiled substrate; lanes 2 and 7, substrate only; lanes 3 and 8, 30 fmol mtMCM as hexamer; lanes 4 and 9, 90 fmol mtMCM as hexamer; lanes 5 and 10, 270 fmol mtMCM as hexamer. D–F, summary of three independent experiments with standard deviation of the percent displacement by the mtMCM (A), spMCM (B), and ecDnaB (C). DNA duplex translocation (I), closed circles; DNA-RNA hybrid translocation (II), open circles.

ecDnaB, mtMCM, and spMCM along ssDNA stimulates their ATPase activity. Stimulation of ATPase activity is therefore indirect evidence for helicase movement along nucleic acids. Thus, an ATPase assay was used to determine whether the helicases are capable of translocation along RNA.

As expected, the ATPase activity of all three helicases is stimulated by ssDNA (Fig. 5, A–C). RNA, on the other hand, does not stimulate ATPase activity; only the basal level of activity was observed with mtMCM and spMCM (Fig. 5, A and B). The ecDnaB protein exhibits a different behavior. Its ATPase activity is inhibited in the presence of RNA (Fig. 5C). The reason for this is not clear. As the MCM and DnaB proteins have different

structures it is possible that upon binding to RNA the ecDnaB structure is locked in a conformation that prevents the intrinsic ATPase activity, the access of the nucleoside to the active site, or dissociation of the ADP after hydrolysis.

The Replicative Helicases Can Translocate along DNA-RNA Duplexes— The replicative helicases of bacteria, archaea, and eukarya are all capable of translocating along duplex DNA (16, 17, 20). The substrates required for dsDNA translocation, however, are different for the three enzymes. Whereas the archaeal MCM can slide onto duplex DNA (16), the eukaryotic MCM requires a 3'-ssDNA overhang (16, 20) and DnaB requires a 5'-ssDNA overhang (17).

After establishing that the helicases cannot move along RNA (Figs. 1–3), their ability to move along a RNA-DNA duplex was determined using substrates similar to those previously used to demonstrate dsDNA translocation by the enzymes (16, 20). It was previously shown that although DnaB and the eukaryotic MCM require a single-stranded overhang region (5' for DnaB and 3' for MCM), the archaeal enzyme can initiate duplex translocation from a blunt end (16, 20). Three oligonucleotides were constructed and hybridized to produce the desired single-stranded and double-stranded regions of DNA or RNA (schematically shown at the top of each panel in Fig. 6). As shown in Fig. 6, all three enzymes are capable of translocating along the RNA-DNA duplex, as is evident from the displacement of the right duplex region (Fig. 6, A–C, lanes 8–10 (II), see also panels D–F).

Translocation along the hybrid duplex is indistinguishable from translocation along duplex DNA (Fig. 5, A–C, compare lanes 8–10 (II) to lanes 3–5 (I), see also panels D–F). It is possible, however, that the bacterial and eukaryal enzymes displace the RNA prior to the displacement of the downstream duplex. This is not the case, however, as neither of the enzymes can unwind DNA or RNA duplexes of 25 nucleotides if not provided with a forked DNA substrate (supplemental Fig. S1, B and C). In addition, all three enzymes have the ability to displace streptavidin (mtMCM) or nitroavidin (spMCM and ecDnaB) from DNA-RNA hybrids (data not shown). These results show that, in contrast to RNA translocation, when the RNA is a part of a

RNA-DNA hybrid duplex the helicase can move along it without major difficulty.

DISCUSSION

The study described here demonstrates that the three replicative helicases studied, representing the three domains of life, are capable of unwinding a DNA-RNA hybrid. What might the *in vivo* role of such activity be? Does the replicative helicase encounter RNA-DNA hybrids during chromosomal DNA replication? A growing number of studies in bacteria and eukarya suggest that such RNA-DNA hybrids (R-loops) do exist on DNA. Therefore, if these structures are not removed from the DNA ahead of the replication fork the replicative helicase has to remove or bypass them.

Studies conducted with bacteria and eukarya demonstrated that under some circumstances RNA-DNA hybrids form as the result of transcription in cells harboring mutations in certain genes. For example, transcription in *E. coli* cells harboring a mutation in topoisomerase I results in stable R-loop RNA-DNA hybrids (21, 22). Similarly, studies in *Saccharomyces cerevisiae* Hpr1 mutant cells show that RNA-DNA hybrid structures are formed during transcription (23). The depletion of the splicing factor ASF/SF2 from chicken cells also results in the formation of RNA-DNA hybrids (24).

Although these and other examples demonstrate that mutations in different genes can result in transcription-mediated RNA-DNA hybrid formation, the base composition of the gene transcribed may dictate whether R-loops are formed. It was shown that when transcription results in long purine-rich stretches of RNA it promotes the formation of RNA-DNA hybrids (25, 26).

RNA-DNA hybrids may also play important roles during normal cell growth. It is well established that dsRNA can regulate gene expression post-transcriptionally via RNA interference. Recently, evidence suggests that short RNA molecules may also directly regulate transcription by regulating cytosine methylation at promoter regions and by chromatin modification (Refs. 27 and 28 and references therein). Although the exact mechanism by which the RNA regulates these cellular process is unknown, it was suggested that it may be mediated by the formation of an R-loop containing RNA-DNA hybrids on the chromosome (27, 29, 30).

Thus, either as a result of mutation or due to normal cell growth, the replicative helicase may encounter RNA along its path during chromosomal replication. As the helicase would be translocating along DNA following assembly at the origin, the data presented here suggest that such RNA on DNA would not inhibit DNA replication as it likely would be removed by the helicase.

The replicative helicases, at least the eukaryotic MCM complex, may also play a direct role during transcription elongation. MCM was shown to interact with the eukaryotic RNA polymerase (7, 8). The MCM complex also interacts with the transcription activator Stat1 (31–33), and interaction with MCM is required for Stat1-mediated transcription (33). It was also found that MCM moves along with RNA polymerase during transcription, and it was suggested that the MCM may unwind

the DNA ahead of the RNA polymerase (33). It is tempting to speculate that the bacterial and archaeal helicases may also have roles in transcription. In addition, in light of the data presented here, it is also possible that, if under certain circumstances the transcript reannealed to the template DNA, the helicase could unwind it behind the RNA polymerase.

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