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Bound Lac repressor protein differentially inhibits the unwinding reactions catalyzed by DNA helicases

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ABSTRACT

A partial duplex DNA substrate containing the Lac repressor binding site, within the duplex region, was constructed to examine the effect of bound Lac repressor on the unwinding reaction catalyzed by several DNA helicases. The substrate contained 90 base pairs of double-stranded DNA and, in the absence of Lac repressor, was effectively unwound by each of the seven helicases tested. The unwinding reactions catalyzed by *Escherichia coli* Rep protein, bacteriophage T4 Dda protein and *E.coli* DNA helicase I were not inhibited by the presence of bound Lac repressor. Both SV40 T antigen and *E.coli* helicase II were partially inhibited by bound repressor at the highest repressor concentrations tested. The helicase reactions catalyzed by *E.coli* DnaB protein and helicase IV were substantially inhibited by the presence of bound protein. When the length of the duplex region was increased to 323 base pairs the inhibition spectrum caused by bound Lac repressor on the unwinding reactions catalyzed by DnaB protein, helicase I and helicase II was essentially the same as that observed using the shorter partial duplex molecule. Inhibition of the unwinding reaction was due to the presence of bound Lac repressor as evidenced by the substantially weaker inhibition of helicase IV by Lac repressor in the presence of IPTG. In addition, we have shown that Rep protein displaces the bound repressor protein during the course of an unwinding reaction.

INTRODUCTION

DNA helicases catalyze the unwinding of duplex DNA by disrupting the hydrogen bonds between base pairs in an energy requiring reaction (for reviews see 1–4). Several DNA helicases have been purified and characterized from a variety of sources ranging from bacteriophage to mammalian cells. Ten different enzymes with DNA helicase activity have been identified in *E.coli* alone (2). Genetic and biochemical studies suggest that these enzymes participate in most, if not all, aspects of DNA metabolism including DNA replication, repair, recombination and bacterial conjugation. The most commonly used direct assay for helicase activity measures the displacement (unwinding) of a DNA fragment that has been annealed onto a circular phage single-stranded DNA (ssDNA) molecule. Although useful in describing the biochemical reaction catalyzed by a helicase, this substrate does not accurately reflect the DNA substrate most likely encountered in the cell. For example, this DNA substrate does not contain long regions of duplex, supercoiled DNA nor are there proteins bound to the DNA. Indeed, most *in vitro* biochemical assays of proteins involved in DNA metabolism are conducted using protein-free DNA. The effect of either nonspecific duplex DNA binding proteins or site-specific DNA binding proteins on the activities of these enzymes remains largely unexplored.

One exceptional study involves bacteriophage T4 DNA replication *in vitro*. Alberts and his colleagues (5, 6) have used this replication system to begin an evaluation of the effect of proteins bound to DNA in the path of the advancing replication fork. For example, an RNA polymerase molecule, bound in the path of the replication fork, inhibited progression of the fork *in vitro*. Actively transcribing RNA polymerase complexes, traveling in the same direction as the DNA replication fork, caused the rate of replication fork movement to slow to that of transcription. Interestingly, the addition of purified phage T4 Dda helicase to this system displaced bound RNA polymerase from the DNA and allowed replication to proceed at normal rates (5). Dda protein has also been shown to allow the phage T4 replication fork to pass through nucleosome-bound DNA without displacing the histone octamers (6). Although the physiological role of Dda protein remains unknown, it is possible that its helicase activity is utilized to allow replication forks to proceed through protein-bound DNA *in vivo*.

Comparable studies in *E.coli* have not been reported. Since DNA replication proceeds at a rate that is ten-fold faster than transcription (7), it is likely that replication forks encounter transcription complexes (for review, see 8). In addition, the replication fork must also replicate regions of the chromosome that are complexed with site-specific DNA binding proteins like the various repressors of transcription. By analogy with the phage T4 system, such potential barriers to replication must be removed

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to allow DNA replication to proceed at a normal rate. Since Dda protein has been reported to be able to perform this function in the T4 replication system, perhaps a similar DNA helicase plays an analogous role in *E.coli* replication.

To initiate an investigation into this possibility, and to determine the effect of a bound protein on the unwinding reaction catalyzed by a DNA helicase, a simple assay was devised. A partial duplex DNA substrate containing the Lac repressor binding site was constructed, and the effect of bound Lac repressor on the unwinding reaction catalyzed by seven DNA helicases was determined. The helicase reactions catalyzed by Rep protein, helicase I and Dda protein were not inhibited by the presence of bound Lac repressor. The helicase reactions catalyzed by either helicase II or the SV40 T antigen were partially inhibited by bound protein and the helicase reactions catalyzed by helicase IV and DnaB protein were substantially inhibited by bound Lac repressor.

MATERIALS AND METHODS

Enzymes

Purified Lac repressor was kindly provided by Dr M.Lewis (University of Pennsylvania) and stored at −20°C in 1 M Tris−HCl (pH 7.6), 40% glycerol and 28 mM β-mercaptoethanol. Aliquots were removed before use, diluted 10-fold in 1 M Tris−HCl (pH 7.5), diluted another 10-fold in glycerol-free buffer containing 0.2 M KCl, 0.2 M Tris−HCl (pH 7.5), 0.3 mM dithiothreitol and 0.1 mM EDTA and stored at 0°C for up to 2 months.

Purified Rep protein, helicase I, helicase II and helicase IV were from this laboratory and purified as previously described (9−12). DnaB protein was provided by Dr E.Bastia (Duke University) and Dr K.Marians (Sloan-Kettering Cancer Institute). Bacteriophage T4 Dda protein was provided by Dr B.Alberts (University of California at San Francisco). SV40 T antigen was provided by Dr B.Stillman (Cold Spring Harbor Laboratories).

*Hpa*II and *Pvu*II restriction endonucleases were from New England Biolabs. Terminal deoxynucleotidyl transferase (TdT), DNase I, T4 polynucleotide kinase and DNA polymerase I (Klenow fragment) were from US Biochemicals, Inc.

DNA and nucleotides

M13mp18 ssDNA and replicative form I (RFI) DNA were prepared as previously described (13). Poly(dI-dC) was from Pharmacia-LKB Biotechnology, Inc. Salmon sperm DNA was from Sigma. [α-^{32}P]dCTP and [γ-^{32}P]ATP were from Amersham Corp. Unlabeled nucleoside 5′-triphosphates were from US Biochemicals.

DNA substrate preparation

The construction of partial duplex DNA substrates has been described (11). To prepare a substrate containing a Lac repressor binding site, M13mp18 RFI DNA was digested to completion with *Hpa*II restriction endonuclease. The 89 base pair (bp) DNA

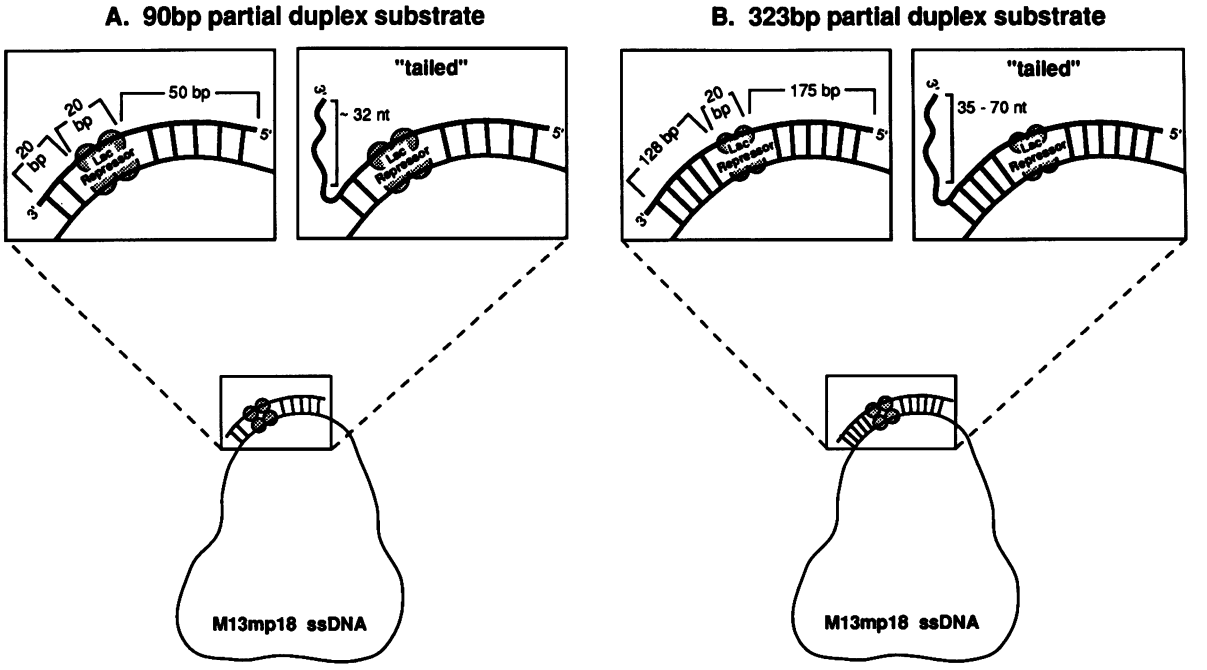

Figure 1. Partial duplex DNA substrates containing the Lac repressor protein binding site. Partial duplex DNA substrates containing a Lac repressor binding site were constructed as described under 'Materials and Methods'. (Panel A) The duplex region of the substrate (enlarged) is 90 bp in length and contains the Lac repressor binding site. The remainder of the molecule contains 7160 nucleotides of M13mp18 ssDNA. Lac repressor is represented covering approximately 20 bp, as inferred from DNase I and chemical footprinting studies (20). Approximately 20 bp of duplex DNA exist on the 3′ side of bound Lac repressor, with respect to the annealed DNA fragment. Approximately 50 bp of duplex DNA are on the 5′ side of bound Lac repressor. The 'tailed' 90 bp partial duplex substrate contains a 3′ poly dC tail 28−37 nucleotides in length. (Panel B) The duplex region of the substrate (enlarged) is 323 bp in length and contains the Lac repressor binding site. The remainder of the molecule contains 6927 nucleotides of M13mp18 ssDNA. Lac repressor covers approximately 20 bp. Approximately 127 bp of duplex DNA exist on the 3′ side of bound Lac repressor; approximately 175 bp of duplex DNA are on the 5′ side of bound Lac repressor. The 'tailed' 323 bp partial duplex substrate contains a 3′ poly dT tail 35−70 nucleotides in length.

fragment containing the Lac repressor binding site was isolated after resolution on a native polyacrylamide gel, electroeluted and the purified fragment was used in annealing reactions with 2 μg of M13mp18 ssDNA to construct a partial duplex DNA substrate. Alternatively, M13mp18 RFI DNA was digested with *Pvu*II, and the 322 bp DNA fragment containing the Lac repressor binding site, purified as above, was used in annealing reactions to construct a 322 bp partial duplex substrate. This longer substrate contained 127 bp on the 3′ side and 175 bp on the 5′ side of the Lac repressor binding site. After annealing the substrate was 3′-end labeled using DNA polymerase I (Klenow fragment) and [α-^{32}P]dCTP, extracted with phenol/chloroform and purified by chromatography on a Bio-Gel A-5 M column (Bio-Rad) as described (11). The resulting 90 bp and 323 bp partial duplex DNA substrates are depicted in Figure 1.

For some experiments the 89 bp *Hpa*II fragment was dephosphorylated using bacterial alkaline phosphatase and 5′-end labeled using polynucleotide kinase and [γ-^{32}P]ATP as described (14). The [^{32}P]DNA fragment was subsequently annealed to M13mp18 ssDNA and the partial duplex substrate was purified by gel filtration. The 90 bp dsDNA fragment used in gel mobility shift assays was prepared by isolating the 89 bp *Hpa*II restriction fragment and 3′-end labeling with DNA polymerase I (Klenow fragment) and [α-^{32}P]dCTP as described (14).

'Tailed' partial duplex DNA substrates containing the Lac repressor binding site were constructed to use in DnaB protein helicase assays (Figure 1). The 90 bp partial duplex substrate was 'tailed' using a modification of the TdT reaction previously described (15). Reaction mixtures (75 μl) contained 100 mM sodium cacodylate (pH 7.0), 1 mM $CoCl_2$, 50 μg/ml bovine serum albumin, 40 μM dCTP, 8.5 units TdT and approximately 13 μM [^{32}P]DNA partial duplex substrate. Reaction mixtures were incubated at 37°C for 60 min and stopped by the addition of 2 μl of 0.5 M EDTA. The volume was increased to 100 μl by the addition of 25 μl of a solution containing 100 mM NaCl/10 mM Tris−HCl (pH 8.0)/1 mM EDTA and the mixture was extracted with equal volumes of phenol and chloroform. The aqueous layer was applied to a Bio-Gel A-5 M gel filtration column (1.5 ml) and fractions containing the labeled substrate, which eluted in the void volume, were pooled. The size of the 3′ tail was estimated by running an aliquot of heat-denatured substrate on a sequencing gel adjacent to a dideoxynucleotide sequencing ladder. The 'tailed' substrate contained a population of DNA molecules extended at the 3′ end by 28−37 nucleotides (shown schematically in Figure 1A). Approximately 50% of the molecules contained a poly(dC) 'tail', as determined by densitometric tracing of autoradiograms from the helicase reactions. The tailed molecules were clearly resolved from molecules lacking a tail on the 6% polyacrylamide gels used to analyze helicase reactions.

The 323 bp partial duplex DNA substrate was 'tailed' in a reaction similar to that described above except that reaction mixtures (100 μl) contained 28 μM [^{32}P]DNA partial duplex substrate, 120 μM TTP and 34 units TdT. Reactions were incubated at 37°C for 8 min, extracted with phenol/chloroform and the 'tailed' DNA substrate was chromatographed on a Bio-Gel A-5M gel filtration column as described above. The 323 bp partial duplex 'tailed' substrate is shown schematically in Figure 1B.

Helicase reaction mixtures using the tailed substrate were identical to the other helicase reaction mixtures except that the concentration of the 90 bp 'tailed' DNA substrate was reduced approximately 3-fold to 670 nM and the NaCl concentration was increased from 25 mM to 35 mM. The concentration of the 323 bp 'tailed' substrate used in helicase assays was approximately 1.75 μM and the NaCl concentration was 35 mM.

Gel mobility shift DNA binding assays

DNA binding assays were performed using the 90 bp 3′-end labeled M13mp18 RFI *Hpa*II restriction fragment. Reaction mixtures (20 μl) were identical to helicase reaction mixtures except that each reaction contained approximately 0.12 μM [^{32}P]DNA fragment and 25 μg/ml poly(dI-dC). Lac repressor was added and the reactions were incubated at 37°C for 5−10 min to allow binding of Lac repressor to its operator. After addition of loading dyes the samples were directly loaded onto pre-electrophoresed 5% polyacrylamide gels as described (16). Gels were electrophoresed at 170 volts for 1.5−2 hours, dried and exposed to X-ray film. The dried gels were quantitated by cutting the gel and counting in a liquid scintillation counter.

DNase I footprint assays

DNase I footprint assays were performed essentially as described (17). The 89 bp partial duplex DNA substrate (2 μM) was incubated with the indicated amounts of Lac repressor at 37°C for 5−10 min in reaction mixtures that were identical to helicase reaction mixtures. After incubation, reaction mixtures were

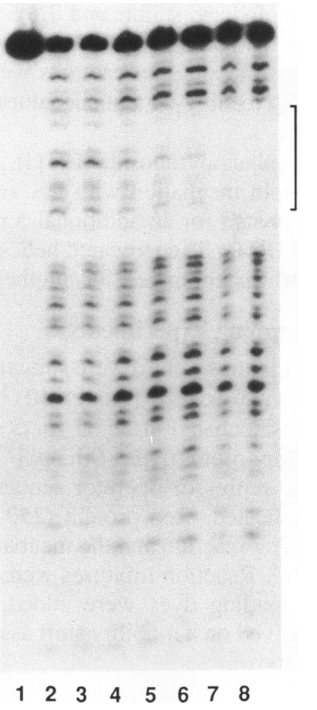

Figure 2. Lac repressor binds to the partial duplex DNA substrate. DNase I footprint assays were performed as described under 'Materiels and Methods' using the 89 bp partial duplex DNA substrate containing a Lac repressor binding site. The substrate was labeled at the 5′ end of the annealed fragment. Lane 1, no Lac repressor and the DNA was incubated in the absence of DNase I; the reaction products shown in lanes 2−8 were obtained after incubation with DNase I as described under 'Materials and Methods'. The following concentrations of Lac repressor tetramer were used: lane 2, 0 M; lane 3, 1×10^{-11} M; lane 4, 1×10^{-10} M; lane, 5, 2×10^{-10} M; lane 6, 4×10^{-10} M; lane 7, 1×10^{-9} M; Lane 8, 1×10^{-8} M. The bracket indicates the region of the partial duplex DNA substrate protected from DNase I digestion by bound Lac repressor.

removed to room temperature and 19 μl of a solution containing 10 mM $MgCl_2$, 5 mM $CaCl_2$, 50 mM KCl and 150 μg salmon sperm DNA was added to each. 1 ng DNase I was added to each reaction, and the samples were incubated at room temperature for 3 min. DNase I digestion was stopped by the addition of 10 μl of a solution containing 0.1 mM EDTA and 3 M ammonium acetate. Nucleic acids were precipitated, after the addition of 4 μg salmon sperm carrier DNA, using 250 μl ice-cold ethanol. The precipitates were collected by centrifugation, washed once with cold 70% ethanol and dried. The pellets were suspended in 4–5 μl of a formamide dye solution containing 95% formamide and 0.05% bromophenol blue and xylene cyanol. Reaction products were resolved on a 6% polyacrylamide sequencing gel containing 7 M urea.

Helicase activity assays

The displacement of a [^{32}P]DNA fragment from the partial duplex helicase substrate was measured as previously described (18). The reaction mixtures (20 μl) were assembled on ice, the indicated amount of Lac repressor was added and the reactions were incubated at 37°C for 5–10 min to allow Lac repressor to bind to its operator site on the DNA substrate. The indicated helicase enzyme was then added, and incubation was continued for 10 min. The amount of each helicase used is indicated in the appropriate figure legend and was chosen to insure that unwinding was in the linear range. The reactions were stopped with the addition of 10 μl of a stop mix containing 38% glycerol, 50 mM EDTA, 1% sodium dodecyl sulfate and 0.1% bromophenol blue and xylene cyanol. In some cases the stop mix also contained 250 μg/ml proteinase K and the reactions were incubated for 5 min at 37°C prior to loading on a nondenaturing polyacrylamide gel.

Isopropyl-β-thiogalactopyranoside (IPTG), when present, was added after the 5 min incubation with Lac repressor. Reaction mixtures were incubated for an additional 5 min at 37°C in the presence of 1 mM IPTG. The indicated helicase was then added and the incubation was continued for another 10 min at 37°C.

Lac repressor trapping experiments

Helicase reaction mixtures (20 μl) were similar to those described above. Lac repressor was added (to 1×10^{-10} M tetramer) and the reactions were incubated at 37°C for 5–10 min. Prior to the addition of Rep protein, the 90 bp [^{32}P]DNA fragment containing the Lac repressor operator sequence was added (to ~0.06 μM) as indicated. Rep protein (250 ng) was added to initiate the unwinding reaction and the incubation was continued for 10 min at 37°C. Reaction mixtures were removed to room temperature, gel loading dyes were added, and the reaction products were resolved on a mobility shift assay polyacrylamide gel as described above.

Other methods

DNA concentrations were determined by directly measuring the absorbance at 260 nm and are expressed as nucleotide equivalents. The concentrations of the helicase substrates and the dsDNA fragment were estimated from the known concentration of DNA in the labeling reactions. A 75% recovery from the gel filtration column was assumed, which represents the average amount of DNA recovered from this column as determined during the original design and implementation of this helicase assay (11). Protein concentrations for helicase I, helicase II, helicase IV and Rep protein were determined by the method of Bradford (19) using bovine serum albumin as the standard. The concentrations of these proteins therefore, may be overestimated by a factor of up to two. The concentrations of Lac repressor, Dda protein, DnaB protein and T Antigen were provided by the laboratory supplying the protein.

RESULTS

A 90 bp partial duplex helicase substrate that binds Lac repressor

A partial duplex DNA substrate containing the Lac repressor binding site within the duplex region was constructed as described under 'Materials and Methods'. A schematic representation of this DNA substrate, with Lac repressor bound as expected from methylation interference data and DNase I footprinting data (for review see 20), is shown in Figure 1A. It is important to note that the Lac repressor binding site is not centered directly on the dsDNA region of the substrate. Instead, the binding site lies toward the 3′-end of the labeled strand. There are approximately 20 bp to the 3′-side of bound repressor protein and approximately 50 bp to the 5′-side. To insure that Lac repressor bound to this substrate at the expected site, DNase I footprint analysis was performed. A partial duplex DNA substrate labeled on the 5′-end of the restriction fragment was used as the substrate for the DNase I footprint shown in Figure 2. Lac repressor protected the expected region of the substrate in a protein concentration-dependent manner.

The partial duplex DNA substrate shown in Figure 1A contained approximately 7000 nucleotides of ssDNA in addition to the 90 bp duplex region. To determine if this excess ssDNA altered the affinity of Lac repressor for its binding site, gel mobility shift assays were performed in the presence and absence of ssDNA (data not shown). The substrate containing the Lac repressor operator sequence was the 90 bp *Hpa*II restriction fragment used in construction of the partial duplex substrate. The presence of 2 μM M13mp18 ssDNA did not alter the affinity of Lac repressor for its binding site. In each case, essentially all of the DNA fragment was bound at Lac repressor concentrations between 1×10^{-9} and 1×10^{-8} M tetramer. Approximately 50% of the fragment was bound at 5×10^{-10} M Lac repressor tetramer.

Bound Lac repressor differentially inhibits the unwinding reactions catalyzed by various helicases

The effect of bound Lac repressor on the unwinding reaction catalyzed by a helicase was examined using the partial duplex DNA substrate described above. Increasing amounts of Lac repressor were bound to the partial duplex substrate prior to incubation with the indicated helicase. The effect of bound Lac repressor on the individual unwinding reactions catalyzed by several helicases which unwind DNA in a 3′ to 5′ direction is shown in Figure 3 (panel A). The unwinding reaction catalyzed by Rep protein was not significantly affected by the presence of bound Lac repressor. At the highest concentration of Lac repressor tested, less than 10% inhibition of the Rep protein unwinding reaction was observed. Under these conditions, all of the partial duplex substrate molecules should contain bound Lac repressor. Similar results were obtained using Rep protein concentrations two-fold higher and lower than indicated (data not shown). SV40 T antigen, on the other hand, was partially inhibited by bound Lac repressor. High concentrations of Lac repressor caused approximately 40% inhibition of the SV40 T

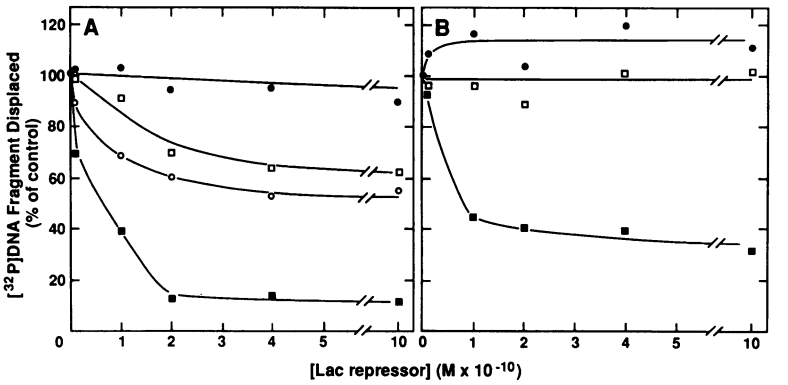

Figure 3. Bound Lac repressor differentially inhibits the unwinding reaction catalyzed by several helicases. Helicase reactions were as described under 'Materials and Methods' using the indicated concentrations of Lac repressor tetramer with the 90 bp partial duplex DNA substrate that contains the Lac repressor binding site. The 'tailed' substrate containing 90 bp of duplex DNA was used with DnaB protein. (Panel A) Quantitative data obtained from reaction mixtures (20 μl) containing helicases which unwind DNA with a 3′ to 5′ polarity. ●, 150 ng Rep protein (110 nM); 100% control unwinding corresponded to 28% displacement of the [^{32}P]DNA fragment. □, 515 ng T antigen (56 nM hexamer); 100% control unwinding corresponded to 59% displacement of the [^{32}P]DNA fragment. ○, 16 ng helicase II (11 nM) 100% control unwinding corresponded to 45% displacement of the [^{32}P]DNA fragment. ■, 10 ng helicase IV (6.7 nM); 100% unwinding corresponded to 51% displacement of the, [^{32}P]DNA fragment. (Panel B) Quantitative data obtained from reaction mixtures (20 μl) containing helicases which exhibit a 5′ to 3′ unwinding polarity. ●, 56 ng Dda protein (57 nM); 100% control unwinding corresponded to 17% displacement of the [^{32}P]DNA fragment. □, 3 ng helicase I (0.8 nM) 100% control unwinding corresponded to 35% displacement of the [^{32}P]DNA fragment. ■, 1 ug DnaB protein (150 nM hexamer); 100% control unwinding corresponded to 64% displacement of a 'tailed' [^{32}P]DNA fragment. The data shown represents the average of at least three experiments. Standard deviations in unwinding assays were approximately 5%, and were never greater than 10%.

antigen unwinding reaction. Helicase II was inhibited slightly more than T antigen. Bound Lac repressor caused approximately 50% inhibition of the unwinding reaction catalyzed by helicase II. Helicase IV was the most severely inhibited of the 3′ to 5′ helicases tested. Even at the lowest concentration of Lac repressor tested, approximately 30% inhibition of the unwinding reaction was observed. Higher concentrations of Lac repressor inhibited the helicase IV unwinding reaction by 90%. When 50-fold more helicase IV was used in the unwinding reaction, the inhibition caused by bound Lac repressor was reduced to 40% at the highest concentrations of Lac repressor (data not shown).

Similar diversity of response to the presence of bound Lac repressor was observed using several helicases that unwind DNA with a 5′ to 3′ polarity (Figure 3, panel B). The unwinding reaction catalyzed by T4 Dda protein was not inhibited by the presence of bound repressor. This result is similar to that reported by others (21). Helicase I was also not inhibited by bound Lac repressor. *E.coli* DnaB protein, however, was substantially inhibited by bound Lac repressor using a 'tailed' 90 bp partial duplex substrate (see Figure 1A and 'Materials and Methods' for description of this substrate). Relatively low concentrations of Lac repressor caused 60% inhibition of the DnaB protein helicase activity. The inhibition increased to approximately 70% with increasing concentrations of Lac repressor. Similar results were obtained using 0.5-fold less DnaB protein in the unwinding reactions (data not shown).

To insure that the inhibition of DnaB protein was not an artifact of the 90 bp 'tailed' partial duplex DNA substrate, a longer substrate was constructed which contained 323 bp of duplex DNA,(see Figure 1B). The Lac repressor binding site was 127 bp from the 3′ end and 175 bp from the end of the annealed DNA fragment on this substrate. The binding of Lac repressor to the 322 bp DNA fragment used to construct this substrate was essentially identical to the binding of Lac repressor to the 90 bp DNA fragment in gel mobility shift assays (data not shown).

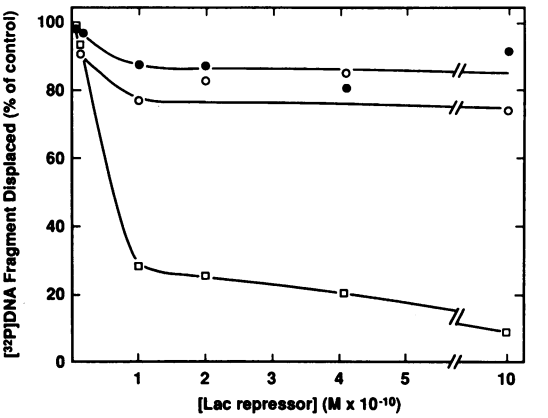

Figure 4. DnaB protein is inhibited by bound Lac repressor using a 323 bp partial duplex substrate. Helicase reactions were as described under 'Materials and Methods' using either the 'tailed', 323 bp partial duplex DNA substrate (DnaB protein) or a 323 bp partial duplex substrate lacking a 3′-tail (helicases I and II). □, 750 ng DnaB protein (120 nM hexamer); ●, 5 ng helicase I (1.4 nM); ○, 80 ng helicase II (49 nM). Each point represents the average of three experiments. 100% control unwinding corresponded to 44−50% displacement of the [^{32}P]DNA fragment in each case.

When increasing amounts of Lac repressor were bound to the partial duplex substrate, the unwinding reaction catalyzed by DnaB protein was profoundly inhibited by Lac repressor (Figure 4). Up to 90% inhibition was achieved with 1×10^{-9} M Lac repressor tetramer. The unwinding reaction catalyzed by helicase I was slightly inhibited using this 'tailed' substrate containing the longer duplex region, and the unwinding reaction catalyzed by helicase II was partially inhibited. The helicase II unwinding reaction was not as strongly inhibited as observed using the shorter partial duplex substrate (compare Figures 3 and 4). This difference may reflect the greater amount of helicase II required

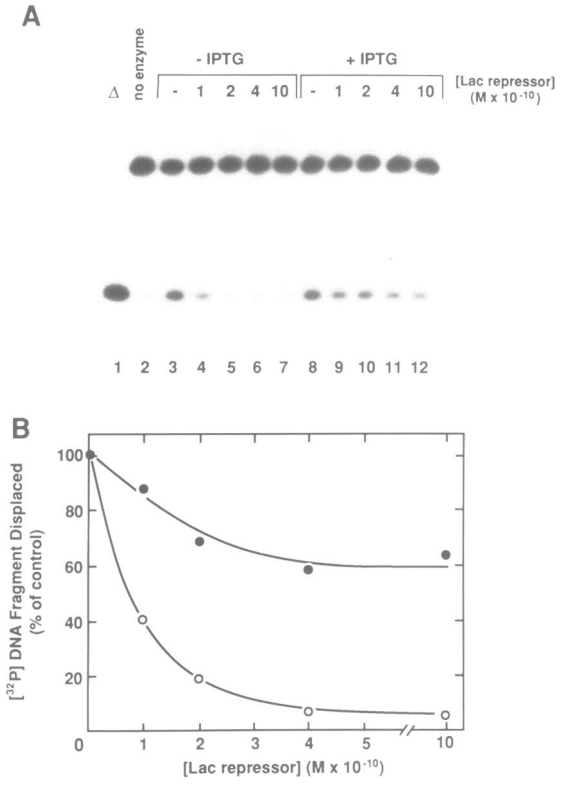

Figure 5. IPTG relieves inhibition of the helicase IV unwinding reaction. Helicase reactions were as described under 'Materials and Methods' using the 90 bp partial duplex DNA substrate. (Panel A) Lane 1, heat denatured control; lane 2, no enzyme control; lanes 3–7 and 8–12, the indicated amounts of Lac repressor tetramer and 10 ng helicase IV (6.7 nM) per 20 μl reaction mixture; lanes 8–12, IPTG was added (to 1 mM) prior to the addition of helicase IV. (Panel B) Data from at least three experiments performed as described in (A) were quantitated as described under 'Materials and Methods'. 100% control unwinding corresponded to 39% displacement of the [^{32}P]DNA fragment. ○, reaction mixtures contained the indicated concentrations of Lac repressor tetramer and 10 ng helicase IV in the absence of IPTG. ●, reaction mixtures contained the indicated concentration of Lac repressor tetramer, 1 mM IPTG and 10 ng helicase IV.

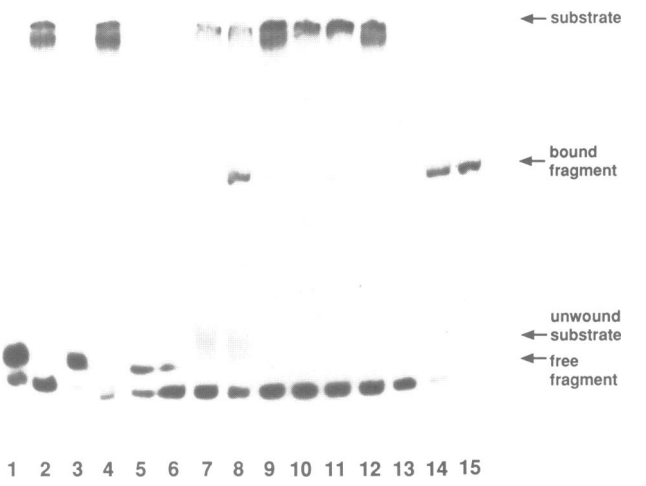

Figure 6. Unwinding by Rep protein releases bound Lac repressor. Helicase reactions were as described under 'Materials and Methods' using 2 μM partial duplex DNA substrate and 0.06 μM 90 bp dsDNA fragment, both of which contain the Lac repressor binding site. Lane 1, heat denatured control with both the partial duplex DNA substrate and the dsDNA fragment; lane 2, no enzyme control containing both the partial duplex DNA substrate and the dsDNA fragment; lane 3, heat denatured control containing the partial duplex DNA substrate; lane 4, no enzyme control containing the partial duplex DNA substrate; lane 5, heat denatured control containing the dsDNA fragment; lane 6, no enzyme control containing the dsDNA fragment; lanes 7–12, reaction mixtures contained both the partial duplex DNA substrate and the dsDNA fragment in addition to the following: lane 7, 250 ng Rep protein (180 nM); lane 8, 1×10^{-10} M Lac repressor tetramer and 250 ng Rep protein; lane 9, 1×10^{-10} M Lac repressor tetramer; lanes 10–12, reaction mixtures and incubation times were identical to lanes 7–9 except that ATP was omitted; lanes 13–15, reaction mixtures were identical to those described for lanes 7–9 except that the partial duplex DNA substrate was omitted. The position to which each labeled DNA species migrated in the gel is noted.

to generate significant unwinding of the longer partial duplex DNA substrate (5-fold more protein). Rep protein and helicase IV were not tested on the longer substrate since they catalyze limited unwinding reactions and cannot unwind a significant fraction of a 323 bp partial duplex in the absence of additional proteins (22). Overall these results are similar to the results observed when Lac repressor was bound to the 90 bp DNA substrate.

Lac repressor was also added to helicase reactions that contained a partial duplex DNA substrate lacking the Lac repressor binding site (data not shown). In this case Lac repressor had no effect on the unwinding reactions catalyzed by either helicase II or helicase IV. The inhibition caused by Lac repressor in reactions containing a DNA substrate with the Lac repressor operator is most likely due to the bound Lac repressor.

To provide additional evidence that inhibition of the unwinding reactions was due to the presence of bound Lac repressor, IPTG was added to a reaction mixture prior to the addition of helicase IV. IPTG binds Lac repressor and reduces the affinity of the repressor for its DNA binding site (20). The addition of IPTG should, therefore, remove at least a portion of the Lac repressor from the DNA substrate and relieve the inhibition caused by the repressor protein block. Since the unwinding reaction catalyzed by helicase IV was severely inhibited by bound Lac repressor, any effect of IPTG on this reaction should be readily apparent. As shown in Figure 5 (see also Figure 3), the unwinding reaction catalyzed by *E.coli* helicase IV was profoundly inhibited by bound Lac repressor. When IPTG was added, subsequent to Lac repressor binding and prior to the addition of helicase IV, the inhibition of the unwinding reaction was substantially relieved. In the absence of IPTG (Figure 5, lanes 3–7), 50% inhibition of the helicase IV, unwinding reaction was observed at a concentration of less than 1×10^{-10} M Lac repressor tetramer. In the presence of IPTG, however, 50% inhibition was not reached using up 1×10^{-9} M Lac repressor tetramer. The addition of IPTG had no effect on the unwinding reaction catalyzed by Rep protein (data not shown).

Lac repressor-DNA complex is disrupted by Rep protein

'Trapping' experiments were performed to determine whether Lac repressor was displaced from DNA by Rep protein, a helicase that was not inhibited by the presence of the bound protein. The strategy employed was as follows: Lac repressor was allowed to bind to the 90 bp partial duplex DNA substrate, and then a labeled dsDNA fragment, also containing the Lac repressor binding site, was added to act as a trap for free Lac repressor. Rep protein was subsequently added to unwind the partial duplex DNA molecules that had been bound by Lac repressor. In this experiment Rep protein is not expected to bind to the dsDNA

fragment since the affinity of Rep protein for dsDNA is much lower than its affinity for ssDNA (23). If Lac repressor is released when Rep protein unwinds the DNA substrate it should be free to bind to the dsDNA fragment also present in the reaction mixtures. This binding event can be detected by observing the gel mobility shift of the dsDNA fragment. Thus, if Rep protein-catalyzed unwinding caused the Lac repressor to be released, then a bound (shifted) dsDNA fragment should be detected only in reactions where Rep protein has unwound the partial duplex DNA substrate.

The experiment presented in Figure 6 shows that the addition of Rep protein displaced bound Lac repressor. In lanes 7–9 the products of reaction mixtures that contained the partial duplex DNA substrate and the labeled dsDNA fragment were resolved on a mobility shift polyacrylamide gel. In the reaction containing Rep protein alone (lane 7), a ssDNA fragment was observed as the product of the unwinding reaction catalyzed by Rep protein. This ssDNA fragment migrates as a smear and more slowly than the heat-denatured fragment because it may have some associated Rep protein (the loading dyes do not contain SDS which is present in helicase reaction stop mix and causes dissociation of the helicase from DNA). None of the dsDNA fragment was bound by Rep protein. In the reaction containing both Rep protein and Lac repressor (lane 8), a significant fraction of the dsDNA fragment was shifted to the position expected for the fragment bound by Lac repressor. The weak band which migrates ahead of the major Lac repressor-shifted band probably represents the weak association between DNA and Lac repressor dimers. In the reaction containing Lac repressor alone (lane 9), very little of the dsDNA fragment exhibited a retarded migration in the absence of Rep protein. Under these reaction conditions, essentially all of the Lac repressor should initially be bound to the partial duplex DNA substrate. The small amount of dsDNA fragment that was bound by Lac repressor, in the absence of Rep protein, may be due to the minor contamination of dsDNA fragment present in the partial duplex DNA substrate preparation. In addition, the $t_{1/2}$ of the Lac repressor-DNA complex may be as short as 5 min (24). Thus, some Lac repressor probably dissociated during the course of the reaction, and was therefore free to bind the dsDNA fragment. The products of reactions performed in the absence of ATP are shown in lanes 10–12. In the reaction containing both Rep protein and Lac repressor (lane 11), there was no increase in the amount of the dsDNA fragment that was bound by Lac repressor. These results suggest that the unwinding reaction catalyzed by Rep protein caused Lac repressor to dissociate from the partial duplex DNA substrate. Control experiments indicate that Rep protein does not bind or unwind the dsDNA fragment (lane 13). In addition, Lac repressor bound the dsDNA fragment in the presence (lane 14) or absence (lane 15) of Rep protein, indicating that the presence of Rep protein did not affect the binding of Lac repressor to its operator site on DNA. These results suggest that as Rep protein unwinds a DNA substrate to which Lac repressor is bound, Lac repressor dissociates and is free to bind the dsDNA fragment which is not a substrate for the helicase.

DISCUSSION

To begin to address the effect of a bound protein on the unwinding reaction catalyzed by a helicase enzyme, four partial duplex DNA substrates were constructed that contained a Lac repressor binding site within the duplex region (see Figure 1). The ssDNA present on the DNA substrate provides an initial binding site for helicases which translocate in either the 5′ to 3′ or the 3′ to 5′ direction. As expected, Lac repressor bound these substrates, under the conditions used in helicase reactions, at the site corresponding to the known Lac repressor operator sequence. It should be noted that the binding site is located asymmetrically within the dsDNA region. Using the 90 bp partial duplex substrates (Figure 1A), helicases that unwind DNA in the 3′ to 5′ direction encounter 50 bp of dsDNA before reaching the region of DNA bound by Lac repressor. Helicases that unwind DNA in the 5′ to 3′ direction encounter approximately 20 bp of dsDNA prior to reaching the region of the substrate bound by Lac repressor. The 323 bp partial duplex DNA substrate (Figure 1B) was constructed so that helicases which unwind DNA in the 3′ to 5′ direction encounter 175 bp of dsDNA before reaching the bound Lac repressor. Helicases that unwind DNA in the 5′ to 3′ direction encounter 127 bp of dsDNA on this substrate before reaching the Lac repressor binding site.

To determine the effect of bound Lac repressor on the unwinding reaction catalyzed by several helicases, the amount of [^{32}P]DNA fragment displaced in unwinding reactions catalyzed by each helicase was quantitated at several concentrations of Lac repressor. Interestingly, the unwinding reactions catalyzed by three of the helicases tested, helicase I, Dda protein and Rep protein, were not inhibited by bound Lac repressor. Each of these enzymes catalyzes a unique unwinding reaction with respect to biochemical mechanism. Dda helicase catalyzes a distributive unwinding reaction in the 5′ to 3′ direction (25). Helicase I catalyzes a processive unwinding reaction in the 5′ to 3′ direction (10). Rep protein catalyzes a very limited unwinding reaction (22) in the 3′ to 5′ direction (26). The absence of an effect of bound Lac repressor, therefore, could not be correlated with unwinding mechanism or polarity. It seems likely that in each case the helicase displaced the bound protein since Lac repressor exhibits a very low affinity for ssDNA (27), the only products of completed unwinding reactions. Nevertheless, experiments were designed to 'trap' displaced Lac repressor released from the partial duplex DNA substrate. The results indicated that the unwinding reaction catalyzed by Rep protein did, in fact, release Lac repressor so that it was free to bind another DNA fragment.

The unwinding reactions catalyzed by two of the helicases examined were partially inhibited by bound Lac repressor. SV40 T antigen and *E.coli* helicase II both catalyze 3′ to 5′ unwinding reactions. the unwinding reaction catalyzed by T antigen is processive (28, 29) whereas the unwinding reaction catalyzed by helicase II is protein concentration-dependent (11). The partial inhibition of these unwinding reactions is difficult to interpret. Helicase II is thought to move unidirectionally along the DNA as it disrupts hydrogen bonds, coating the ssDNA products of the unwinding reaction. In fact, protein tracks have been observed by electron microscopy (30, 31). If helicase II remains stably bound on partially unwound substrate molecules, then transient dissociation of Lac repressor, under conditions of equilibrium, may eventually allow helicase II to move through the duplex region. In this way some fraction of the helicase II unwinding reactions may proceed to completion. In a similar manner, the processive T antigen might be stably bound to the DNA so that it too can proceed through the duplex region upon dissociation of Lac repressor. Since the $t_{1/2}$, of the Lac repressor–operator complex is thought to be relatively short (24), it would be interesting to examine the effect of bound Lac repressor on the

unwinding reactions catalyzed by helicase II or T antigen with shorter incubation periods. If fewer Lac repressor molecules dissociated during the course of the unwinding reaction, greater inhibition of the unwinding reactions might be observed.

The last two helicases examined, helicase IV and DnaB protein, were substantially inhibited by bound Lac repressor. Again, these two helicases share little biochemical similarity. DnaB protein catalyzes a processive unwinding reaction in the 5′ to 3′ direction (32). This enzyme has a direct role in DNA replication and is thought to be the helicase present at the replication fork (for review, see 2). Helicase IV, on the other hand, catalyzes a very limited unwinding reaction in th 3′ to 5′ direction (12). The physiological function of helicase IV is not known. Inhibition of the unwinding reactions catalyzed by helicase IV and DnaB protein suggests that neither of these enzymes is able to displace bound Lac repressor. Moreover, both helicases may dissociate upon encountering the protein block. Transient dissociation of Lac repressor, in this case, would not allow completion of the unwinding reactions.

The range of effects on unwinding reactions produced by bound Lac repressor may reflect on the physiological role of a specific helicase in the cell. For example, Dda protein may be required for the efficient progression of the T4 replication fork (5). The unwinding reaction catalyzed by Dda protein was not inhibited by bound Lac repressor, consistent with previous reports (21). Similarly, the unwinding reaction catalyzed by helicase I, which is required for unwinding of the F plasmid during bacterial conjugation (33), was not inhibited by a bound repressor protein. Thus helicase I may efficiently move through regulatory proteins which might be routinely bound on DNA to be transferred. The role which Rep protein plays in the cell is not known. However, it is known to be involved in bacteriophage DNA replication and has been implicated in chromosomal DNA replication (34–37). Since the unwinding reaction catalyzed by Rep protein was not inhibited by the presence of bound Lac repressor, Rep protein may be capable of displacing proteins bound on the *E.coli* chromosome. DnaB protein, on the other hand, was unable to unwind DNA bound by a repressor protein. Yet, DnaB protein is thought to be the replicative helicase. Since the DNA in the cell is presumed to be associated with many proteins, an additional helicase, such as Rep protein, may be required to allow the replication fork to proceed through regions of DNA bound by proteins. The absence of this activity might result in the slowed replication fork movement observed in *rep* mutants (38–40). Preliminary data suggest that Rep protein can remove bound Lac repressor and allow DnaB protein to unwind the 323 bp partial duplex substrate (unpublished observations).

Recent work by Hiasa and Marians (41) has shown that the effect of bound Tus protein on the unwinding reaction catalyzed by DnaB protein depends on the position of the bound protein with respect to the end of the duplex region first unwound by DnaB protein. The effect of Tus protein on unwinding reactions catalyzed by various helicases may represent a specialized example since the physiological role of Tus protein may be to arrest replication fork movement at the terminus region (42). The experiments reported in this manuscript indicate that the effect of Lac repressor on the DnaB protein unwinding reaction is not dependent on the position of Lac repressor within the duplex DNA region. However, it is possible that the unwinding reaction catalyzed by DnaB protein, as part of a replication complex, is not inhibited by the presence of bound proteins. Further experiments which examine the effect of bound proteins on replication fork movement are required to address this question.

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