Enzyme-catalyzed DNA unwinding: Studies on Escherichia coli rep protein

(cistron A protein/DNA-binding protein)

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ABSTRACT Replication in vitro of the replicative form (RF) I DNA of bacteriophage φX174 requires the phage-induced cistron A (cisA) protein, the host rep protein, DNA-binding protein, ATP, and DNA polymerase III plus replication factors. The rep protein is a single-stranded DNA-dependent ATPase. In this paper we show that φX174 RF I DNA cut by the cisA protein acts as a duplex DNA cofactor for the rep protein ATPase activity, provided that DNA-binding protein is present. In this latter reaction the duplex DNA is unwound by the rep protein with concomitant hydrolysis of ATP. The extents of ATP hydrolysis, DNA unwinding, and, where appropriate, DNA synthesis are proportional to the amounts of DNA-binding protein present. Two ATP molecules are hydrolyzed per base pair unwound. We propose that the obligatory requirement for the cisA protein in the unwinding of φX174 RF I DNA is not simply due to its endonuclease activity but rather is due to its presence at a site for the binding of the rep protein. The rep protein in the presence of DNA-binding protein, but in the absence of cisA protein, unwinds duplex DNA when one strand extends to generate a single-stranded leader region preceding the duplex. We show that rep protein translocates along the leader single strand in a 5'-to-3' direction only and then invades the duplex DNA. The rep protein shows a directional specificity for translocation and unwinding. A model is presented to explain the mechanism of DNA unwinding catalyzed by the rep protein.

The Escherichia coli rep protein is a single-stranded DNA-dependent ATPase that is required for the replication in vitro (1–2) and in vitro (3–6) of replicative form (RF) I DNA of several bacteriophages. Detailed studies of the replication in vitro of φX174 RF I have shown a requirement for the bacteriophage cistron A (cisA) protein and the E. coli DNA-binding protein (DBP), as well as the rep protein, for DNA unwinding in the presence and absence of DNA synthesis (5, 7, 8). It has been suggested that the cisA protein and rep protein form a complex that unwinds DNA, using ATP as an energy source, and that DBP binds to the single strands produced to reduce the rate of rewinding (9). We have shown (5) that the ability of the rep protein to catalyze an ATP-dependent unwinding of duplex DNA does not depend upon the presence of cisA protein, provided that a single-stranded "leader" precedes the duplex region.

In this report, we extend our previous observations and analyze the DNA unwinding reaction biochemically, measuring the stoichiometry of ATP hydrolyzed per base pair unwound. The interaction between DBP and rep protein and the involvement of DBP in strand separation is studied. Employing the reaction in which the rep protein catalyzes unwinding of partial duplexes in the absence of cisA protein, we show that unwinding proceeds unidirectionally in accordance with our previous prediction (5). These results give an insight into the roles played by the individual components of the unwinding reaction and suggest a model to explain the observations. The model also suggests other experiments to elucidate the mechanism further.

MATERIALS AND METHODS

Preparation of DNA and Proteins. φX174 single-stranded DNA was prepared by phenol extraction of purified φX174 am3 bacteriophage. Unlabeled and labeled φX174 RF I DNA was prepared as described by Godson and Vapnek (10). φX174 RF II DNA was made by the partial digestion of RF I by pancreatic DNase I. The cisA protein was purified from φX174 am3-infected E. coli H562 to final specific activity of 2×10^4 units per mg (5). The rep protein was purified from E. coli JFS-19 cells containing the CoE1 plasmid pLC 44-7 to final specific activity of 2×5×10^4 units per mg (5). DNA polymerase III and replication factors (DNA polymerase IIIβ and co polymerase IIIγ) were purified according to Wickner et al. (11) and DBP was purified as described (12).

Complementation Assay for DNA Replication. The complementation assays used for the purification of the cisA protein and rep protein have been described elsewhere (5).

Replication Assay with Purified Components and DNA-Dependent ATPase Assay. These assays have been described (5).

DNA Unwinding Assay. The assay for the unwinding of duplex DNA measured the formation of acid-soluble material by S1 nuclease after the unwinding reaction was over (5). The first incubation (unwinding) contained 0.1 ml: 20 mM Tris-HCl, 4 mM dithiothreitol, 12 mM MgCl₂, 2 mM ATP, and 750 pmol of φX174 RF I (3H)DNA, 5 units of cisA protein, 12 units of rep protein, and 3 μg of DBP. After 15 min at 30°C, an equal volume of 4 M NaCl was added and the incubation was continued at 30°C for 5 min. This was followed by the addition of 15 μg of denatured calf thymus DNA, 0.8 ml of S1 nuclease digestion buffer containing 30 mM sodium acetate at pH 4.5, 1 mM ZnSO₄, 5% (vol/vol) glycerol, and 100 units of S1 nuclease. After incubation at 45°C for 45 min, the reaction was terminated by the addition of 5% trichloroacetic acid and the acid-insoluble radioactivity was determined.

Preparation of Substrates for Determining Directionality of Unwinding. The substrate for 5'-to-3' unwinding was prepared by digesting φX174 RF I ([3H]DNA with Hph I restriction enzyme and separating the products on a 1.4% agarose slab gel. The largest fragment (7=1655 base pairs (bp)) was eluted from the gel, concentrated by adsorption to and subsequent elution from hydroxyapatite, and then annealed with (+) strands of φX174 DNA. Fragment bound to (+) strands was removed by Bio-Gel A-15m gel filtration. The partial duplex molecule was then digested with restriction enzyme Hpa I.

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Abbreviations: RF, replicative form; cisA, cistron A; bp, base pairs; DBP, DNA-binding protein; φX, bacteriophage φX174.
which cuts once in the duplex region to give a linear partial duplex with a 1218 bp duplex region at the (+) strand 3' end, and a 437 bp region at the 5' end. The substrate for 3'-to-5' unwinding was constructed as described above, except that φX174 RF I [32P]DNA was initially digested with restriction enzyme Hpa I and fragment 2 (~1318 bp) was isolated. This was annealed with φX174 (+) strand, and the partial duplex was cut with Hph I to yield a linear partial duplex, with a duplex region of 1288 bp at the 5' end of the (+) strand and 50 bp at the 3' end. The preparations of these substrates are shown in Fig. 1.

Partial Duplex Unwinding Assay. This assay was essentially as described above; each reaction mixture contained 1300 pmol of (+) strand and 300 pmol of fragment hybridized with (+) strand. Incubations were at 30°C for 20 min.

FIG. 1. Schematic representation of the preparation of unwinding substrates to test the directionality of unwinding. Hph I and Hpa I are restriction enzymes; dashed arrows show sites of enzyme cleavage.

FIG. 2. Time course of ATP hydrolysis with φX RF I-cisA complex (2 nmol), rep protein (30 units), DBP (6 μg), and [14C]ATP. Aliquots were removed and the ATP hydrolyzed was determined (○). After 60-min incubation, either rep protein (15 units) (●) or more φX RF I-cisA complex (2 nmol) and DBP (6 μg) (●) were added.

Isolation of Complex Formed between φX174 RF I and cisA Protein. This complex was formed by digesting φX174 RF I [3H]DNA with cisA protein for 30 min at 30°C, followed by neutral 5–20% sucrose gradient centrifugation for 3 hr at 4°C in a Beckman SW 50.1 rotor at 50,000 rpm.

RESULTS
Cofactor Requirements of the rep Protein ATPase Activity. The rep gene product was purified from E. coli cells, using a complementation assay that measured the phage cisA protein and φX174 RF I DNA-dependent DNA synthesis in an extract prepared from rep - cells. In agreement with the previous results of ourselves and others (5, 6), the rep protein is a single-stranded DNA-dependent ATPase. Although double-stranded DNA is not a cofactor for the ATPase activity, φX174 RF I DNA cut by phage cisA protein is a cofactor in the presence but not the absence of the E. coli DBP (see Table 3). The cisA protein introduces a single nick into the (+) strand of the phage RF I DNA and remains covalently bound to the 5' end of the nick (9). It is this complex (φX RF I DNA-cisA) that acts as cofactor for the rep protein ATPase activity (5, 6). The φX RF I DNA-cisA complex was isolated by sucrose gradient sedimentation and the kinetics of ATPase activity catalyzed by rep protein using this cofactor was studied (Fig. 2). ATP hydrolysis was linear with time, reaching a limit after 60 min, the addition of more rep protein at this time did not result in further ATP hydrolysis, whereas the addition of more cofactor did. This suggests that the limit of hydrolysis represents a depletion of active cofactor.

The ATPase activity observed by using cut RF I cofactor DNA as substrate is completely dependent on the DBP (Fig. 3). This is in contrast to the ATPase activity that is observed by

<table>
<thead>
<tr>
<th>Table 1. Inhibition of rep protein ATPase by DBP</th>
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<tr>
<td>DBP, μg</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.15</td>
</tr>
<tr>
<td>0.30*</td>
</tr>
<tr>
<td>0.75</td>
</tr>
<tr>
<td>1.50</td>
</tr>
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</table>

Standard reaction mixtures contained single-stranded φX174 DNA (0.025 μg), rep protein (3 units), and DBP as shown. Incubations were at 30°C for 30 min.

* DNA equivalence.
employing single-stranded DNA as a cofactor. The ATPase activity of the rep protein can be completely abolished when the single-stranded DNA cofactor is complexed completely with DBP. These results are summarized in Table 1. The rep protein is able to bind to single-stranded DNA but is unable to do so when the DNA is complexed with DBP (data not shown).

**DBP Dependency of rep Protein ATPase Activity.** Because the φX RF I DNA-cisA complex is a duplex DNA cofactor for ATP hydrolysis catalyzed by rep protein, the involvement of DBP in this reaction was analyzed. This reaction is DBP dependent, and the extent of hydrolysis is proportional to the amount of DBP added (Fig. 3, Table 2).

The conditions that are optimal for ATP hydrolysis are those that are also essential for promoting DNA synthesis using the cut RF I cofactor (see Table 2). This suggests that the ATP hydrolysis is associated with generation of a suitable template for DNA synthesis—i.e., conversion of a duplex DNA to an unwound form. The unwinding of cut RF I DNA by the rep protein has been demonstrated directly (5, 7).

**DNA Synthesis and DNA Unwinding In Vitro.** We have reported a biochemical assay for measuring DNA unwinding (5), and, using this assay, which measures the formation of single-stranded DNA, we have demonstrated the rep protein-mediated unwinding of φX174 RF I DNA. Previous studies by ourselves and others (5, 7) demonstrated that φX174 RF I DNA-dependent synthesis in vitro is catalyzed by E. coli DNA polymerase III and replication factors, DBP, rep protein, and cisA protein. Table 3 shows that in the absence of DNA synthesis duplex DNA is unwound and that the requirements for DNA unwinding and synthesis are similar. Moreover, there is good correlation between the extent of unwinding and the extent of DNA synthesis.

**Stoichiometry of ATP Hydrolysis and DNA Unwinding.** A time course of DNA unwinding is shown in Fig. 4. The extent of the reaction is dependent upon the amount of DBP added (Fig. 5). Because the extents of both DNA synthesis and ATP hydrolysis are also dependent upon the amount of DBP added (Fig. 3, Table 2), an estimation of the stoichiometry of ATP hydrolysis per base pair unwound can be made (Table 2). A value of 2 ATP molecules hydrolyzed per base pair unwound is obtained, which is in agreement with estimates made by others, in which ATP and dATP hydrolysis at various rep protein concentrations in the synthesis reaction was measured (8). It is also clear from these data that there is a coupling between DNA unwound, ATP hydrolyzed, and DNA template available.

**Partial DNA Duplex Unwinding by rep Protein.** In an attempt to understand the roles of the cisA protein, rep protein, and DBP in the unwinding reaction, the ability of the rep protein to unwind partially duplex φX174 DNA was studied. Partial duplexes were constructed by annealing a ³²P-labeled restriction fragment of less than unit length with φX174 single-stranded DNA circles, and unwinding was assayed by the biochemical unwinding assay. This assay measures the formation of single-stranded DNA as judged by S1 nuclease sensitivity. Partially duplex φX174 DNA was unwound by the rep protein in a DBP-dependent, but cisA protein-independent reaction (Table 4, Fig. 6). Unwinding of the partial duplexes

### Table 2. Stoichiometry of ATP hydrolyzed per base pair unwound

<table>
<thead>
<tr>
<th>DBP, µg</th>
<th>Synthesis</th>
<th>Unwinding</th>
<th>ATP hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>&lt;5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>0.03</td>
<td>10</td>
<td>25</td>
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</tr>
<tr>
<td>0.08</td>
<td>26</td>
<td>78</td>
<td>70</td>
</tr>
<tr>
<td>0.15</td>
<td>41</td>
<td>94</td>
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<tr>
<td>0.30</td>
<td>64</td>
<td>118</td>
<td>112</td>
</tr>
<tr>
<td>0.75</td>
<td>64</td>
<td>146</td>
<td>120</td>
</tr>
<tr>
<td>1.50</td>
<td>84</td>
<td>157</td>
<td>122</td>
</tr>
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</table>

Details of the reaction conditions are shown in legends to Figs. 4 and 5.

### Table 3. Requirements for φX174 RF I DNA replication and unwinding

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Synthesis</th>
<th>Unwinding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>71</td>
<td>157</td>
</tr>
<tr>
<td>-cisA protein</td>
<td>3.6</td>
<td>&lt;8</td>
</tr>
<tr>
<td>-rep protein</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>-DBP</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>-ATP</td>
<td>12</td>
<td>&lt;8</td>
</tr>
<tr>
<td>-RF I, +RF II</td>
<td>&lt;4</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

DNA synthesis and unwinding were measured in separate reaction tubes, but under identical conditions. Incubations were for 40 min at 30°C.

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**Fig. 4.** Time course of DNA unwinding. The reaction mixture contained φX174 RF I [³²P]DNA-cisA protein complex (2 nmol), rep protein (6 µg), and ATP (2 mM). Aliquots were removed at the times shown, and the S1 nuclease-sensitive DNA was determined.

**Fig. 5.** Extent of DNA unwinding at different DBP concentrations. The reaction mixtures contained φX174 RF I [³²P]DNA-cisA protein complex (400 pmol), rep protein (6 µg), DBP as shown, and ATP (2 mM). The DNA unwound was determined after incubation for 80 min at 30°C.
by the rep protein is only observed at binding protein amounts less than stoichiometric with the single-stranded DNA present. As shown above for ATPase activity, complete complexing of single-stranded DNA with DBP prevents rep protein attachment to the DNA. These results suggest that the two proteins compete for the same "sites" on single-stranded DNA. In spite of this limitation, approximately 30% of the input labeled DNA can be unwound (Fig. 6). By eliminating the need for the cisA protein, we are able to conclude that the rep protein in combination with DBP is able to catalyze the complex unwinding of duplex DNA.

**Polarity of rep Protein Catalysis of Partial Duplex Unwinding.** The polarity of rep protein-catalyzed DNA unwinding was studied by using the partial duplex unwinding reaction. In the φX174 RF I unwinding reaction, in which the cisA protein becomes covalently attached to the 5' end, unwinding proceeds in such a way that the 5' end is displaced. With this polarity, the cut 3' end is able to serve as a primer for DNA synthesis. It was of interest to see if the polarity of displacement is an inherent characteristic of the rep protein. If so, it implies that the protein can translocate along DNA and in a predetermined direction. Accordingly, partial linear duplexes were prepared (see Materials and Methods) in which a 32P-labeled strand (approximately 1200 bases long) was annealed with the 3' end of a linear molecule (approximately 9000 bases long) (the flush end of the duplex, which is not an unwinding substrate, has the 5' end labeled). The substrate with the opposite polarity, in which the 32P-labeled strand was annealed to the 5' end of the linear strand, is identical in polarity to the cut φX174 RF I substrate (the 5'-labeled end is internal) (Fig. 1). As is seen in Fig. 6, the rep protein catalyzes the unwinding of only the latter molecule. Thus we conclude that the rep protein is able to bind to the single-stranded portion of the partial duplex molecule and translocate along it in the 3' → 5' direction until a duplex portion is reached. The 5' end of the duplex is then displaced.

**DISCUSSION**

We have previously demonstrated (5) that the rep protein as purified by its essential role in the replication of φX174 RF I DNA in vitro has a single-stranded DNA-specific ATPase activity and plays a central role in the DNA unwinding reaction. The results presented here show that: (i) Stoichiometric amounts of E. coli DNA binding protein inhibit the cofactor activity of single-stranded DNA. (ii) φX RF I DNA-cisA complex isolated by sucrose gradient sedimentation is a cofactor for the rep protein ATPase activity in a reaction that requires E. coli DBP. (iii) The extents of ATP hydrolysis, DNA unwinding, and DNA synthesis are determined by the DBP added, and the stoichiometry of unwinding to ATP hydrolysis is 2 ATP molecules per base pair unwound. (iv) rep protein unwinds partially duplex DNA in the presence of less than stoichiometric amounts of E. coli DBP. Stoichiometric and greater amounts of DBP completely inhibit unwinding. (v) rep protein shows a directionality of unwinding; partial duplexes are only unwound by displacing the 5' end of a duplex.

Several DNA-dependent ATPases have been isolated from E. coli cells (13–16), and some of these are capable of unwinding duplex DNA. Among these, the 75,000-dalton protein resembles the rep protein quite closely. This protein at high enzyme concentrations is capable of unwinding duplexes in the absence of DBP; at low concentrations no unwinding is observed (15). However, this protein has not been shown to complement a rep− extract for φX174 RF I replication in vitro, and it may not be the rep protein.

The results presented here suggest a model to explain how rep protein unwinds DNA at the expense of ATP. The model is based on one main assumption. From studies on the binding of DBP to single-stranded DNA, it is suggested that the protein binds electrostatically to the phosphate backbone of only single-stranded DNA; no appreciable binding to double-stranded DNA is observed (17, 18). We suggest that DBP has many phosphate-binding sites (at least three) and that these are oriented in space such that they are capable of binding the phosphate when DNA is in a single-stranded conformation but not when it is in the double-stranded conformation. We propose that rep protein has similar binding properties to the DBP and hence discriminates between double- and single-stranded DNA on the basis of the phosphate backbone conformation. It follows from this that DBP inhibits the cofactor activity of single-stranded DNA by competing with rep protein for the same DNA-binding sites. These properties are shown in Fig. 7. The phosphates on the DNA backbone are shown schematically as being in a linear relationship, and are further apart on single- than double-stranded DNA. rep protein and DBP have their phosphate binding sites spaced such as to allow single- but not double-stranded DNA binding.

We suggest that, for the rep protein, the phosphates are able to exchange with one another as shown, thereby effecting translocation or net movement along single-stranded DNA. This movement may or may not require energy. We further suggest that when the rep protein has several sites occupied by DNA phosphate, it undergoes an ATP-mediated conformational
change. Specifically, the binding site at the head of the molecule (towards the 3′ end of the DNA chain) becomes related to the next binding site (towards the tail of the molecule) by the same relationship as the phosphates are related in the backbone of double-stranded DNA. We propose that this conformational change requires the binding of ATP and that completion of the cycle requires hydrolysis of the bound ATP and the return of the rep protein to its original conformation. This explains how the rep protein functions as a single-stranded DNA-dependent ATPase (Fig. 7).

Because the rep protein can unwind partially duplex DNA but not fully duplex DNA, we propose that a single-stranded leader region, adjacent to the duplex, is required for rep protein binding. We also suggest that the rep protein shows a net translocation towards the junction of the duplex and single-stranded DNA in a 3′-to-5′ direction, until the foremost phosphate-binding site is unoccupied. The binding of ATP results in a conformational change such that the first phosphate binding site of the rep protein can bind the first phosphate group in the duplex DNA backbone. Upon hydrolysis of the bound ATP, the rep protein returns to its initial conformation, forcing that phosphate out of the configuration compatible with duplex DNA and into that compatible with single-stranded DNA. Hence the duplex is unwound by one base pair. This process is then repeated to lead stepwise DNA unwinding (Fig. 8). If the translocation step leaving one unoccupied binding site at the head of the molecule requires the hydrolysis of one ATP molecule, and the conformational change in the rep protein requires the hydrolysis of one ATP molecule, then the stoichiometry of ATP hydrolysis per base pair unwound would be 2 as determined experimentally.

Although we have no definitive proof of our suggested model and it is not a unique interpretation of our data, we feel that it is in agreement with data presented here; namely that rep protein unwinds DNA unidirectionally, binds to single- but not to double-stranded DNA, hydrolyzes 2 ATP molecules per base pair unwound, and acts catalytically rather than stoichiometrically. It is curious that when αX RFI DNA-cisA complex is the cofactor for ATPase activity, DBP is required. Our model predicts that the rep protein be able to invade duplex DNA from a single-stranded leader region, in the absence of DBP. We have suggested that the cisA protein provides a site analogous to the single-stranded leader, hence allowing rep to bind to the nicked duplex DNA; perhaps DBP is also involved in the provision of this site or the initiation of unwinding of this complex. Alternatively, DBP may be actively involved in the unwinding reaction. This may be by the formation of a protein-protein complex with rep, as has been observed for DNA polymerase.

Alternatively, DBP may be essential to bind to the strand being displaced or to bind "behind" the rep protein. Our model would be supported if it could be shown directly that ATP and DNA result in a reversible conformational change of the rep protein.

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