Purification of the rep Protein of Escherichia coli

AN ATPase WHICH SEPARATES DUPLEX DNA STRANDS IN ADVANCE OF REPLICATION

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The product of the rep gene of Escherichia coli catalytically separates φX174 duplex DNA strands in advance of their replication, utilizing ATP in the process (Scott, J. F., Eisenberg, S., Bertsch, L. L., and Kornberg, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 193-197). The enzyme has now been purified to near-homogeneity. Relatively large quantities were obtained from ColEl-plasmid-containing cells in which the enzyme level was 7 to 10 times above wild type. The assay for rep protein was based on its essential role, with phage-induced estron A protein, in enzymatic synthesis of phage φX174 (+) strands, using duplex circular DNA as template. The enzyme exhibits a molecular weight of 65,000 under denaturing and reducing conditions. The turnover number of the enzyme is approximately 6800 ATP molecules/min in strand separation as measured by extent of replication, or in an uncoupled reaction using single-stranded DNA effector.

The product of the rep gene of Escherichia coli is required for replication of the duplex replicative form of φX174 phage and for subsequent synthesis of progeny viral single strands. It is not required in conversion of the infecting viral single strand to RF’ (1-4). Mutations in the rep locus are not lethal for replication of the duplex replicative form of 4x174 phage DNA. Mutations in the rep locus are not lethal for replication of the duplex replicative form of 4x174 phage DNA.

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Mid-bearing strain which makes 7 to 10 times more rep protein than wild type. In this paper we also describe the purification of rep protein from this strain (in a yield of 30 mg from 4.6 kg of cells), its enzymatic behavior, and some of its physical properties. The ATPase functions are reported in greater detail in the succeeding paper (9).

MATERIALS AND METHODS

Bacterial and Phage Strains

These were generously provided as follows: E. coli HF4704 rep3 (thy (at 37°C), rep3) by Dr. S. Eisenberg of this laboratory; RLM365 [rha, lys, thyA (50 μg/ml), polB, str'] by Dr. R. McMacken of this laboratory; HF4704 [thy (at 37°C), Su'] and phage φX174 am3 by Dr. R. Sinsheimer (California Institute of Technology); M0766 (recB2, rec22, sbcB15, thi, endE) by Dr. M. Oishi (Public Health Research Institute of the City of New York); and phage G4 by Dr. N. G. Godson (Yale University). Construction of JFS19 [rha, lys, thyA (50 μg/ml), polB, str'/φX174]ColE1 (pLC44-7) lvo', cyA', rho', rep'] is described in this paper. The rha' marker on pLC44-7 was detected by Daniel Oppenheim (Stanford University).

Preparation of Colicin

Colicin was prepared from W3110/ColEl cells as described previously (10) except that the cell suspension in the salt wash buffer was sonicated for 1 min before removing cells and debris by centrifugation. The ammonium sulfate precipitate containing colicin was dissolved in buffer containing 50% (v/v) glycerol to a concentration of 8 x 10^2 units/ml and stored at -20°C.

Plasmid Transfer to Construct ColEl-rep Hybrid Strains

Plasmid donor and recipient strains were grown to mid-log phase (A600 ≈ 0.5) on a shaker at 37°C. During the last 30 min of growth donor strains were removed from the shaker and kept at 37°C for maximum growth of F- pilus. Recipient cells (0.2 ml) and donor cells (0.02 ml) were mixed and rotated slowly (40 rpm) at 37°C for 45 min to permit mating to occur. Addition of colicin (0.2 ml of L + Thy broth described below) containing 2 units/ml of colicin) followed by a 90-min period on a rotary shaker at 37°C, killed cells without ColEl plasmids. A drop of the mixture was plated on L + Thy + methyl methanesulfonate (400 μg/ml) to counterselect M0766 (recA donor), or on minimal medium M63 (11) containing thymine (2 μg/ml) to counterselect RLM365 or JFS19 donors (lys and high thymine (50 μg/ml) requirements). Plates were allowed to dry, and streaked to yield single colonies. Clones surviving either of the sequences of two counterselections were presumed to be successful recipients of the ColEl and F plasmids. Such clones were verified to be colicin-resistant and then tested for rep' phenotype by spotting G4 phage (10^6 pla) on a soft-agar layer containing the clone on an L + Thy plate (see below). HF4704 rep' served as a positive control; HF4704 rep3' (mapping at 83 min on the revised E. coli map (12)) containing
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ColE1/E. coli hybrids with a his\textsuperscript{+} fragment of lac\textsuperscript{+} fragment (mapping at 44 and 8 min, respectively) served as negative controls.

**Media; Growth and Storage of Cells**

Cells used for genetic manipulation were grown in L + thy broth, L broth (13) containing 50 \( \mu \)g/ml of thymine. For solid media, 1.5% agar was added for plates, or 0.7% for soft agar. Colicin plates were prepared fresh before use by adding 25 units of colicin per plate in 4 ml of L + thy + 1.5% agar (melted and then cooled to 50°C before adding colicin). JFS91 cells for enzyme preparations were grown in 70-liter batches in a 100-liter New Brunswick Permacel in media containing per liter: 10 g of yeast extract (Difco or Ardamine Z), 10 g of Cereolose (w/v), 0.05 g of thymine, 0.01 g of thiamin, 1.85 g of K\textsubscript{2}HPO\textsubscript{4}, and 10 g of K\textsubscript{2}PO\textsubscript{4}. Cereolose (w/v) and thiamin were sterilized separately and added to the Permacel medium after sterilization and cooling. Seventy liters of media were inoculated with 8 liters of a 14-h culture of cells grown in four 6-liter Erlenmeyer flasks on a rotary shaker at 37°C. Medium used for inocula was the same, except that the yeast extract was exclusively Difco and colicin (1000 units/liter) was added when cultures were started. Each 2-liter culture was inoculated with 0.25 ml of starter culture, which had been grown to stationary phase in L + thy broth containing 1 unit/ml of colicin, and had been maintained at 2°C - 4°C for a minimum of an equal volume of sterile glycerol. The pH in the Permacel was maintained between 6.8 and 7.1 by addition of 50% NaOH; the level of foam was controlled by using a minimum possible amount of SAG 471 antifoam (no more than 3 ml/liter). Cells were harvested at A\textsubscript{600} = 8 to 10 by centrifugation in a Sharples continuous flow centrifuge. Yields of cell paste were 900 to 1000 g wet weight. Cells were resuspended in A\textsubscript{600} = 400 in Buffer A (see below) and frozen by pouring them slowly into liquid nitrogen. The frozen pellets were stored at -20°C in waxed cardboard cartons. HMS83 and M0676 cells were grown and stored in the same manner except that no colicin was added to the inocula.

**Chemicals**

Sources were as follows: Bio-Rex 70, acrylamide, bisacrylamide, and dithiothreitol, Bio-Rad; Tris, Sigma; bovine albumin (crystaline), Miles; thiamin (grade B), spermidine-Cl (grade A), and calf thymus DNA (grade A), Calbiochem; DEAE-cellulose DE 52, Whatman; Sephadex G-25 Medium, Pharmacia; ammonium sulfate (ultrapure), sucrose (enzyme grade), and thymine, Schwarz/Mann; [\( ^{32} \)P]ATP and [\( ^{3} \)H]dATP, New England Nuclear; [\( ^{3} \)H]thymidine, Amersham or Schwarz/Mann; Cereolose, Corn Products; Ardamine Z Yeast Extract; Yeast Products; Difco Yeast Extract, Difco; agar, Pfizer; SAG 471 antifoam, Union Carbide; Brij 58, Pierce; and 2-mercaptoethanol, Eastman. All other chemicals were reagent grade purchased from Sigma.

**Enzymes**

Sources were as follows: egg white lysozyme, Worthington; \( \alpha \)X174 ciaA protein (60,000 daltons) was more than 90% pure as judged by SDS-polyacrylamide gel electrophoresis (4.4 x 10\(^{4}\) units/mg of protein) (14); DNA polymerase III holoenzyme (DEAE-Sephadex peak Fraction V, 5.9 x 10\(^{4}\) units/mg of protein) was approximately 60% pure (15); E. coli DBP (15) was a homogeneous preparation (17).

**Buffers**

Buffer A contained 50 mM Tris-Cl (pH 7.5) and 10% sucrose (w/v); Buffer B, 50 mM Tris-Cl (pH 7.5), 20% glycerol (v/v), 1 mM EDTA, and 5 mM dithiothreitol; Buffer C, 50 mM imidazole-Cl (pH 6.6), 20% glycerol, 1 mM EDTA, and 5 mM dithiothreitol; Buffer D, 25 mM imidazole-Cl (pH 6.6), 20% glycerol, 1 mM EDTA, and 5 mM dithiothreitol. Additions of salts were as indicated below. All pH measurements were made at 23°C.

**Assays for rep Protein**

A. RF Replication by Complementation of Crude Enzyme Fraction from rep Cells (18) – Fraction II from HF4704 rep3 cells (uninfected or infected with \( \Delta Xams \)) were prepared as described previously (18). The reaction mixture, in 25-\( \mu \)l volume, contained: 50 mM Tris-Cl (pH 7.5), 6% sucrose (w/v), 10 \( \mu \)M dithiothreitol; Buffer B, 25 mM imidazole-Cl (pH 6.6), 20\% glycerol, 1 mM EDTA, and 5 mM dithiothreitol. Additions of salts were as indicated below. All pH measurements were made at 23°C.

**Preparation of DNA-cellulose**

SS calf thymus DNA-cellulose was prepared as described previously (21); it was stored as a dry powder at -20°C. Fines were removed from the powder by suspension and settling in Buffer C containing 50 mM NaCl. The column of adsorbant was equilibrated by washing with several bed volumes of Buffer B containing 100 mM NaCl until the A\textsubscript{260} value reached that of the buffer.

**Preparation of Antibody against rep Protein**

Antiserum was raised against rep protein in a 6-month-old virgin female White New Zealand rabbit by administration of one 150-\( \mu \)g and two 100-\( \mu \)g injections of the protein with Freund's complete adjuvant, in the foot pads, at 1-week intervals. Four weeks after the first injection, bleedings of approximately 25 ml from the ear vein were begun and taken at 1-week intervals. Each bleeding was preceded 4 or 5 days earlier by an injection of 10 \( \mu \)g of rep protein intravenously. Serum from three such bleedings were pooled and the IgG fraction was prepared by precipitation with 40% saturation ammonium sulfate, followed by transfer to 20 mM potassium phosphate buffer, pH 7.0, on a G-25 Sephadex column, and passage through a DE52 DEAE-cellulose column equilibrated with the same buffer.

**RESULTS**

Construction of ColE1-1 rep Hybrid Strain which Overproduces rep Enzyme — ColE1/E. coli hybrid plasmids from the

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Production of rep protein by ColE1-1 rep hybrid cells</td>
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<tr>
<td>Fraction II preparations were obtained as described in Table I legend and rep was assayed as under Assay B (&quot;Materials and Methods&quot;).</td>
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<tr>
<td>Source of Fraction II</td>
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<tr>
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<tr>
<td>MO576 (no plasmid)</td>
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<tr>
<td>HMS80 (no plasmid)</td>
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<tr>
<td>JF519 (ColE1-1 rep hybrid)</td>
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Clarke and Carbon colony bank known to carry markers close to rep at 83.5 min in the E. coli chromosome (22) were transferred to HF4704 rep3 cells. Clones so obtained were tested for complementation of the rep defect (see "Materials and Methods").

Of five cya+ plasmids tested, only pLC44-7, known to carry both ilu+ and cya+ markers, complemented the chromosomal rep deficiency. The plating efficiency of G4 phage on HF4704 to rep+ at 83.5 min in the E. coli standard plaque assay (data not shown). The pLC44-7 plasmid was both ilu+ and rep+ (see "Materials and Methods"). Replication and ATPase assay values were multiplied by 2.5 to correct the Step VI specific activity to that obtained in an optimum reaction. I. Extract: Frozen cell suspension (900 to 1200 g) was thawed at 2°C or less (all subsequent operations were at 4°C or less; centrifugations were in a Beckman JA-14 rotor at 0°C) and diluted with Buffer A to A578 = 200 (2 x 10^11 cells/ml). After the pH was adjusted to 8.0 with Tris base, a solution (1/10 volume) containing 0.18 M spermidine - Cl, 50 mM EDTA, and 1.5 M ammonium sulfate (to produce a more tightly compacted pellet of cell debris after centrifugation) and lysozyme dissolved in Buffer A was added (200 μg/ml final concentration). The mixture was stirred, transferred to centrifuge bottles, and kept on ice for 45 min. After warming in a 37°C bath for 4 min with swirling and mixing by inversion at 1-min intervals, the bottles were chilled on ice, and centrifuged for 1 h at 13,000 rpm, frozen in liquid nitrogen, and stored at -70°C for several weeks without loss of activity. Five such pellets representing a total of 4650 g cells were thawed, each dissolved in 0.1 of its respective Fraction I volume of Buffer C, and pooled (Fraction II, 710 ml). III. DEAE-cellulose and BioRex 70: Figure II was diluted with Buffer D to a conductivity equivalent to Buffer C containing 93 mM ammonium sulfate added to each milliliter of buffer) and centrifugation at 13,000 rpm for 30 min. The pellet was washed by suspension (using a glass tissue homogenizer) in 0.1 of the Fraction I volume of Buffer B (containing 0.1 x NaCl and 0.24 g of ammonium sulfate added to each milliliter of buffer) and centrifugation at 13,000 rpm for 30 min, frozen in liquid nitrogen, and stored at -70°C for several weeks without loss of activity. Five such pellets representing a total of 4650 g cells were thawed, each dissolved in 0.1 of its respective Fraction I volume of Buffer C, and pooled (Fraction II, 710 ml). III. DEAE-cellulose and BioRex 70: Fraction II was diluted with Buffer D to a conductivity equivalent to Buffer C containing 93 mM ammonium sulfate (equal to 200 mM NaCl), applied to a 1250-ml DE 52 column (9 x 23 cm) equilibrated in Buffer C + 93 mM ammonium sulfate. The column was washed with 800 ml of the same buffer, the void volume (500 ml) discarded, and the next 1550 ml collected as a single fraction. After dilution in Buffer D (to a conductivity equivalent to Buffer C + 50 mM NaCl), the sample was loaded on a 1250-ml Bio-Rex 70 column (7.5 x 28 cm) equilibrated in Buffer C + 50 mM NaCl. The column was washed with 2500 ml of Buffer C + 250 mM NaCl, and eluted in 125-ml fractions with 2500 ml of Buffer C + 400 mM NaCl. The activity eluted later than the protein peak in Buffer C + 400 mM NaCl elute (Fig. 1A). Peak fractions were pooled and concentrated with ammonium sulfate (0.35 g/ml). Pellets, frozen in liquid nitrogen and stored at -70°C, were redisolved in Buffer B to a conductivity equivalent to Buffer B + 100 mM NaCl (Fraction III). IV. DNA-cellulose: Fraction III (465 ml) was loaded on a 90-ml DNA-cellulose column (3.7 x 8 cm) equilibrated in Buffer B + 100 mM NaCl. The column was washed with 90 ml of Buffer B + 100 mM NaCl and 270 ml of Buffer B + 250 mM NaCl, then eluted in 45-ml fractions with 270 ml of Buffer B + 100 mM NaCl (Fig. 1B). Active fractions were pooled and dialyzed for 8 to 10 h against a volume of Buffer B calculated to dilute the pool to the conductivity of Buffer B + 25 mM NaCl. A precipitate, formed during dialysis was removed by centrifugation; its removal from the supernatant (Fraction IV) is essential to eliminate two contaminants which would otherwise persist after Step V. V. DEAE-cellulose: Fraction IV was loaded on a 30-ml DEAE-cellulose column (2.5 x 6.5 cm) equilibrated in Buffer B + 25 mM NaCl. The column was washed with 30 ml of Buffer B + 25 mM NaCl and 90 ml of Buffer B + 50 mM NaCl, then eluted in 15-ml fractions with 150 ml of Buffer B + 75 mM NaCl. The bulk of the activity was eluted as a sharp concentrated peak with the 75 mM NaCl eluant front (Fig. 1C). Pooled peak fractions (Fraction V) frozen in liquid nitrogen and stored at -70°C were stable at least 8 months; however, losses were incurred on freezing and thawing. Fraction V at 0.1 mg of protein/ml or greater was relatively stable at 0°C. Preliminary experiments indicate that the enzyme is stabilized by the presence of 50 to 60% glycerol. 

Clarke and Carbon colony bank known to carry markers close to rep at 83.5 min in the E. coli chromosome (22) were transferred to HF4704 rep3 cells. Clones so obtained were tested for complementation of the rep defect (see "Materials and Methods").

Table II

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Replication assay</th>
<th>ATPase assay</th>
<th>ATPase replication</th>
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<tr>
<td></td>
<td>mg</td>
<td>Total activity</td>
<td>Specific activity</td>
<td>Total activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>units x 10^9</td>
<td>units x 10^6/mg</td>
<td>units x 10^9</td>
</tr>
<tr>
<td>I Lysis</td>
<td>87,000</td>
<td>90</td>
<td>18</td>
<td>450</td>
</tr>
<tr>
<td>II Ammonium sulfate</td>
<td>21,000</td>
<td>78</td>
<td>37</td>
<td>260</td>
</tr>
<tr>
<td>IIIa DEAE-cellulose I</td>
<td>15,000</td>
<td>48</td>
<td>36</td>
<td>170</td>
</tr>
<tr>
<td>IIIb Bio-Rex 70</td>
<td>580</td>
<td>30</td>
<td>2300</td>
<td>75</td>
</tr>
<tr>
<td>IV DNA-cellulose</td>
<td>79</td>
<td>18</td>
<td>6300</td>
<td>54</td>
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<tr>
<td>V DEAE-cellulose II</td>
<td>30</td>
<td>14</td>
<td>4700</td>
<td>29</td>
</tr>
</tbody>
</table>

Clarke and Carbon colony bank known to carry markers close to rep at 83.5 min in the E. coli chromosome (22) were transferred to HF4704 rep3 cells. Clones so obtained were tested for complementation of the rep defect (see "Materials and Methods").

Of five cya+ plasmids tested, only pLC44-7, known to carry both ilu+ and cya+ markers, complemented the chromosomal rep deficiency. The plating efficiency of G4 phage on HF4704 rep3F* pLC44-7 was the same as on rep* HF4704 in a standard plaque assay (data not shown). The pLC44-7 plasmid was then transferred from the MV1217F* host by F-mediated transfer to RLM365, a polA+, lac+, rep+ derivative of HMS83 (see "Materials and Methods") in order to place the plasmid in a polA+ host which is capable of supporting ColEI replication and also ly ses well. The strain JFS19, so derived, which is rep+ on the chromosome as well, was shown to contain the plasmid by transfer to HF4704 rep3, and testing of progeny clones for rep+ phenotype as before. JFS19, MO676, and HMS 83 cells were grown, extracts prepared, and levels of rep activity measured (see "Materials and Methods"). Rep protein activity was 7- to 10-fold greater in extracts of JFS19, the plasmid-carrying strain, than in extracts from HMS83 or MO676 (Table I).

Purification of rep Protein from Strain JFS19 – A procedure was devised which yielded 30 mg of rep protein from 4650 g of strain JFS19 cells, with an overall recovery of 15% (Table II) (Fig. 1). A soluble protein extract was prepared by gentle heat lysis after treating the cells with lysozyme. The cell debris was removed by centrifugation in the presence of spermidine and 150 mM ammonium sulfate in the lysate. The proteins were then fractionated by ammonium sulfate precipitation, and residual nucleic acid fragments were removed by passage of the protein fraction containing the bulk of the rep protein activity through DEAE-cellulose. The proteins were further fractionated by chromatography on Bio-Rex 70. A nearly pure preparation of rep was then obtained by binding to DNA-cellulose from which the rep protein activity was eluted using 1 M NaCl. Final purification of rep protein was achieved by (i) dialysis of the DNA-cellulose fraction against a buffer containing 25 mM NaCl; (ii) removal of an insoluble precipitate which

Purification of E. coli rep Protein

Formed during dialysis; and (iii) chromatography on DEAE-cellulose.

Purity and Physical Properties of rep Protein from JFS 19 - In addition to the coincidence of sharp protein and activity peaks on DEAE-cellulose chromatography (Fig. 1c), the protein migrated as a single band on SDS-polyacrylamide gel electrophoresis at acrylamide concentrations of 6% (Fig. 2) and 10% (data not shown). No individual impurities, at levels of greater than 1% were observed. Relative to standards on the 6% gel, the rep protein migrated as a polypeptide of 65,000 daltons under denaturing and reducing conditions. The protein has a sedimentation coefficient (S_{20,w}) of approximately 4.9 to 5.0 S as measured in a glycerol gradient relative to markers of DNA polymerase I, hemoglobin, and cytochrome c (2). The enzyme has a turnover number of 6800 ATP molecules hydrolyzed/min based on the specific activity of Fraction V rep protein (Table II), and in strand separation as measured by extent of replication (see succeeding paper (9)).

Purification of rep Protein of Strain HMS 83 - To compare rep protein coded by the hybrid plasmid in strain JFS19 with that coded by the E. coli chromosome, the enzyme was purified from 1 kg of HMS83 cells yielding 1.4 mg of rep protein (greater than 95% pure). The procedure was similar to that for JFS19 (Table II), except that cells were lysed with 0.1% Brij-58 detergent and the DEAE-pass through step (IIIa) was omitted. The enzymes prepared from JFS19 and HMS83 showed the same molecular weight on an SDS-polyacrylamide gel.

Pure rep Protein Fully Complements rep Mutant Extracts - Fraction V rep protein prepared from JFS19 was used to complement DNA replication by extracts of rep-deficient cells (Assay A, "Materials and Methods"). The extent of incorporation was 1.4 times that obtained with a less pure fraction. The complete complementation of rep extracts by the pure protein indicates that this enzyme is the sole defective component of such cells.

Antibody Prepared against rep Protein Inhibits Both Replication and ATP Hydrolysis in Coupled Reaction - Antibody against rep protein was prepared and assayed for inhibitory activity against rep in Assays B and C (see "Materials and Methods"). In Assay B (based on reconstituted dX viral strand synthesis dependent on rep) strong and concomitant inhibition of both replication and ATP hydrolysis was observed when rep IgG was incubated with the rep protein for 10 min at 0°C prior to addition to the reaction mixture. About 50% inhibition of both replication and ATP hydrolysis was observed in reactions containing 3.8 ng of rep with 1.0 μg of rep antibody, or 76 ng of rep with approximately 13 μg of rep antibody. Control IgG prepared against dnaB protein produced no inhibition but rather about 10% stimulation under the same conditions. Surprisingly, the same protein ratios resulted in at most 10%
The two analyses were in good general agreement, and both when purified from either the overproducing strain (JFS19) or present. Such residues have not as yet been positively identified.

When compared with the uncoupled ATPase reaction from the standard strain (HMS83) used routinely in this laboratory. Sedimentation of protein was also analyzed by Dr. Marian Koshland at the University of California, Berkeley."

Amino acid analysis was performed by AAA Laboratory, Mercer Island, Washington, on a Durrum Analyzer model D-500. Fraction V rep protein was dialyzed extensively against distilled water, lyophilized, and subjected to a 24-h 6 M HCl hydrolysis at 110°C. The value obtained for serine and threonine were increased by 10% and 5%, respectively, to compensate for destruction by acid. Half-cystine was determined on a sample which was performic acid-oxidized prior to acid hydrolysis. Tryptophan was determined on a sample which was hydrolyzed with alkali. Numbers of residues per mol of protein monomer were calculated assuming a molecular weight of 65,000.

Amino acid composition of rep protein

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>moles/65,000 g</th>
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<tbody>
<tr>
<td>Alanine</td>
<td>45.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>41.9</td>
</tr>
<tr>
<td>Aspartic acid, asparagine</td>
<td>54.1</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>3.5</td>
</tr>
<tr>
<td>Glutamic acid, glutamine</td>
<td>80.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>31.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>11.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>26.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>72.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>31.4</td>
</tr>
<tr>
<td>Methionine</td>
<td>13.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>24.9</td>
</tr>
<tr>
<td>Proline</td>
<td>17.9</td>
</tr>
<tr>
<td>Serine</td>
<td>29.7</td>
</tr>
<tr>
<td>Threonine</td>
<td>31.4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>5.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>18.8</td>
</tr>
<tr>
<td>Valine</td>
<td>25.7</td>
</tr>
</tbody>
</table>

The rep protein was first partially purified on the basis of its ability to complement rep' extracts for in vitro replication of φX RF (18). Subsequently, the partially purified rep protein was assayed by its complementation of three other purified proteins and RF I as template in a reconstituted reaction for the extensive net synthesis of φX viral (+) circles (19, 20). This assay was later applied for the entire purification procedure of rep protein. The pure enzyme obtained by this procedure fully complements rep' extracts for φX RF replication. Thus, rep protein is the only required component absent from such deficient extracts, and is, therefore, what was cautiously termed the rep-dependent protein (18). That rep protein is in fact the product of the rep gene is further substantiated by overproduction of the enzyme by the E. coli strain bearing extra copies of a fragment of DNA mapping at the rep genetic locus contained in the ColEl/E. coli hybrid plasmid.

The rep protein shown previously to be an ATPase dependent on single-stranded DNA (20) is a member of a growing family of such E. coli enzymes. These include a 75,000-dalton protein (23-25), dnaB protein (26, 27), protein Y required for replication (28), a 180,000-dalton DNA unwinding enzyme I (29, 30), rep B-C nucleace (31), type I restrictionendonucleases (e.g. EcoK12, EcoB, EcoFI) (32), and T-phage-induced enzymes; the latter include a T4-induced 50,000-dalton protein (33), the T4 gene 44-62 and 45 products needed in replication (34) and the T7 gene 4 protein with primase and strand-separation functions (35, 36). Among these, the 75,000-dalton protein bears the closest resemblance to rep protein in its physical features and ATPase properties. However, the denatured polypeptide size is clearly larger than that of rep protein; the strand-separating activity associated with it has not been shown to be catalytic or interactive with citron A protein, or to be the product of the rep gene (24, 25). Within the family of single-stranded DNA-dependent ATPases, it is impressive that most are known to effect strand separation of duplex DNA or have a role in DNA replication. Instances of ATPases, as those found in eukaryotic virus cores (37), may prove to have such replicative functions upon further study.

Among general mechanisms that may be considered for coupling energy of ATP hydrolysis to the catalytic separation of DNA strands by rep protein, one is distributive (nonprocessive), and the other is processive. In a distributive mechanism (Fig. 3) a rep ATP complex binds at the opening of a DNA fork causing the melting of a base pair (I); hydrolysis of ATP (II) enables rep-ADP to dissociate from the fork and DNA binding protein to attach to it (III) thus keeping the strands separated; rep-ATP is regenerated (IV) by a displacement of ADP from rep by ATP.

In a processive mechanism (Fig. 4), rep protein remains bound at the unzipping fork. Once the ternary complex of rep, ATP, and DNA fork is formed (I), hydrolysis of ATP and melting of a base pair ensue (II), followed by attachment of DBP (III), and a replacement of ADP by ATP (IV).

The stoichiometries in both schemes remain vague. Measurements of ATP hydrolysis during replication of φX RF clearly indicate 2 molecules of ATP (or dATP) consumed for each nucleotide polymerized and, by inference, each base pair melted (9). However the number of rep protein subunits involved is not known and the binding of a DBP tetramer is
complicated by the fact that it occupies a DNA stretch of about 40 nucleotides (17).

In a choice between these two types of mechanisms, the processive kind seems far more attractive. A reaction which needs to be repeated successively many thousands of times on the same template, as in the case of replication, would be more plausibly designed to be processive. In addition, biochemical and electron microscopic studies of strand-separation and replicative intermediates, in vitro, demonstrate a sustained association of proteins at the replicating fork (14) which would likely include cistron A and rep proteins. The preference which rep protein exhibits for a cistron A protein-complexed replicating fork as a DNA effector for ATPase action (9) suggests an active role for rep protein in the maintenance of a highly ordered protein-DNA complex. Further studies are needed to collect the crucial facts required to establish the correct mechanism of the ATP-driven unwinding of the replicating fork.

Acknowledgment—We are grateful for the excellent technical assistance of T. Trobough on the large scale preparation of rep protein.

REFERENCES