THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 253, No. 9, Issue of May 10, pp. 3292-3297, 1978
Printed in U.S.A.

Purification of the *rep* Protein of *Escherichia coli*

AN ATPase WHICH SEPARATES DUPLEX DNA STRANDS IN ADVANCE OF REPLICATION*

(Received for publication, October 25, 1977)

JOHN F. SCOTT AND ARTHUR KORNBERG

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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 ColE1-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 *cistron* A protein, in enzymatic synthesis of phage ϕX174 (+) strands, using duplex circular DNA as template. The protein 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] (1-4). Mutations in the *rep* locus are not lethal for the host cell, but block RF replication of phages ϕX174 (1), M13 and fd (5), G4 (6), and P2 (7). These mutations also result in: larger cell size, more DNA per cell, slightly lower ratio of DNA to cell mass, more replicating forks per chromosome, slower fork movement, and a faster sedimenting nucleoid body containing more DNA (5, 8).

Wild type *E. coli* contains only about 50 copies of *rep* protein per cell. Therefore, to obtain pure protein for enzymatic and physical characterization of the enzyme, we selected a plasmid-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).

* This work was supported in part by grants from the National Institutes of Health and the National Science Foundation. This is Paper 5 in the series "An Enzyme System for the Replication of Duplex Circular DNA: The Replicative Form of Phage ϕX174." Paper 4 is Ref. 14. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: RF, replicative form DNA; RF I, covalently closed, circular superhelical ϕX DNA; SS, single-stranded DNA; DBP, *E. coli* DNA binding protein; holoenzyme, DNA polymerase III holoenzyme; SDS, sodium dodecyl sulfate; *rep*, *rep* protein; albumin, bovine serum albumin.

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, thy*A (50 μg/ml), *polB, str*^{r}], HMS83 [*rha, lys, thy*A (50 μg/ml), *polA, polB, lac, str*^{r}] W3110/ColE1 and MV12 (*lacY*, Δ*trp*E5, *thr, leu, recA*/F$^+$/pLC44-7) 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); MO676 (*recB21, recC22, sbcB15, xthl, endl*) 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), *pol*B, *str*^{r}/F$^+$/ColE1 (pLC44-7) *ilv*$^+$, *cya*$^+$, *rho*$^+$, *rep*$^+$] is described in this paper. The *rho*$^+$ marker on pLC44-7 was detected by Daniel Oppenheim (Stanford University).

Preparation of Colicin

Colicin was prepared from W3110/ColE1 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 8×10^3 units/ml, and stored at −20°C.

Plasmid Transfer to Construct ColE1-rep Hybrid Strains

Plasmid donor and recipient strains were grown to mid-log phase ($A_{595} \simeq 0.5$) 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-pili. 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 ColE1 plasmids. A drop of the mixture was plated on L + Thy + methyl methanesulfonate (400 μg/ml) to counterselect MV12 (*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) requirers). 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 ColE1 and F plasmids. Such clones were verified to be colicin-resistant and then tested for *rep*$^+$ phenotype by spotting G4 phage (10^6 pfu) 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

ColE1/*E. coli* hybrids with a *his*$^+$ fragment of *lac*$^+$ 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 μg/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). JFS19 cells for enzyme preparations were grown in 70-liter batches in a 100-liter New Brunswick Fermacel in media containing per liter: 10 g of yeast extract (Difco or Ardamine Z), 10 g of Cerelose, 0.05 g of thymine, 0.01 g of thiamin, 1.85 g of KH_2PO_4, and 10 g of K_2HPO_4. Cerelose (80%, w/v) and thiamin were sterilized separately and added to the Fermacel 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 stored at −20°C after addition of an equal volume of sterile glycerol. The pH in the Fermacel was maintained between 6.8 and 7.1 by addition of 50% NaOH; the level of foam was controlled by adding the minimum possible amount of SAG 471 antifoam (no more than 3 ml/70 liters). Cells were harvested at A_{595} = 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 to A_{595} = 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 MO676 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 (crystalline), 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; [α-^{32}P]ATP and [^{3}H]dTTP, New England Nuclear; [^{3}H]thymidine, Amersham or Schwarz/Mann; Cerelose, 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 from Baker.

Enzymes

Sources were as follows: egg white lysozyme, Worthington; φX174 *cisA* protein (60,000 daltons) was more than 90% pure as judged by SDS-polyacrylamide gel electrophoresis (4.4×10^6 units/mg of protein) (14); DNA polymerase III holoenzyme (DEAE-Sephadex peak Fraction V, 5.9×10^5 units/mg of protein) was approximately 60% pure (15); *E. coli* DBP (16) 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 (v/v), 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 φX*am3*) were prepared as described previously (18). The reaction mixture, in 25-μl volume, contained: 50 mM Tris·Cl (pH 7.5); 6% sucrose (w/v); 10 mM dithiothreitol; 0.1 mg/ml of albumin; 5 mM $MgCl_2$; 50 μM each of dATP, dCTP, and dGTP; 18 μM [^{3}H]dTTP (600 cpm/pmol); 800 μM ATP; 100 μM each of CTP, UTP, and GTP; 2 mM spermidine·Cl; 40 μg of Fraction II protein from uninfected *E. coli* HF4704 *rep3*; 10 μg of Fraction II protein from φX am3-infected *E. coli* HF4704 *rep3*; 800 pmol (total nucleotide) of φX RF I DNA; and *rep* protein to be assayed. After 20 min at 30°C, the reaction was stopped by addition of 0.2 ml of 0.1 M sodium pyrophosphate and 1 ml of 10% (w/v) trichloroacetic acid. The precipitate was collected on glass fiber filters (Whatman GF/C), washed three times with 1 M HCl, 0.1 M sodium pyrophosphate, twice with 95% ethanol, dried, and counted in 5 ml of a toluene-based scintillation fluid in a liquid scintillation counter. One unit of *rep* (complementing) activity is defined as 1 pmol of total nucleotide incorporated/min. Activity of *rep* protein is linear with amount of *rep* protein added over a 10-fold range of *rep* units added but yields apparent activities of only about 1% that determined by Assays B and C.

B. Synthesis of Viral Strands from RF in Reconstituted System (19, 20) – The 25-μl reaction mixture contained: 50 mM Tris·Cl (pH 7.5); 15% (v/v) glycerol; 10 mM dithiothreitol; 0.1 mg/ml of albumin; 5 mM $MgCl_2$; 50 μM each of dATP, dCTP, and dGTP; 18 μM [^{3}H]dTTP (150 cpm/pmol); 200 to 800 μM ATP; 200 pmol (total nucleotide) of φX RF I DNA; 500 units of φX *cisA* protein; 1.5 μg of DBP; 16 units of holoenzyme; and *rep* protein to be assayed. Incubation was at 30°C for 15 min and treated as above. One unit of *rep* (recon) activity is defined as 1 pmol of total nucleotide incorporated/min. Although the values were linear over a 10-fold range with the amount of added *rep* protein, the absolute values varied up to 4-fold depending on the *cis*A protein and holoenzyme preparations. For this reason, each set of assays included pure *rep* protein (47×10^6 units/mg) as a standard.

C. ATPase Dependent on SS DNA – This assay relied on the release of [^{32}P]ADP from [α-^{32}P]ATP as described elsewhere (20) using a 10-μl reaction mixture. One unit of ATPase is defined as 1 pmol of ADP formed/min.

Preparation of Bio-Rex 70

Bio-Rex 70 (200 to 400 mesh), Na^+ was converted to H^+ form with HCl, washed with water, equilibrated with 20% (v/v) glycerol, titrated with imidazole base, washed with 20% (v/v) glycerol, and equilibrated with respect to conductivity in Buffer C containing 50 mM NaCl.

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 2 M NaCl. The column of adsorbant was equilibrated by washing with several bed volumes of Buffer B containing 100 mM NaCl until the A_{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-μg and two 100-μg 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 μg 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-rep Hybrid Strain which Overproduces rep Enzyme – ColE1/*E. coli* hybrid plasmids from the

TABLE I

Production of rep protein by ColE1-rep hybrid cells

Fraction II preparations were obtained as described in Table II legend and *rep* was assayed as under Assay B ("Materials and Methods").

Source of Fraction II	Activity	Specific activity
	units/g cell paste	*units/mg protein*
MO676 (no plasmid)	51,000	19,000
HMS83 (no plasmid)	85,000	13,000
JFS19 (ColE1-*rep* hybrid)	587,000	190,000

TABLE II

Purification of rep protein from ColE1-rep hybrid cells

Replication and ATPase assays were Assays B and C ("Materials and Methods"). Replication 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 A_{595} = 200 (2×10^{11} cells/ml). After the pH was adjusted to 8.0 with Tris base, a solution (1/9 volume) containing 0.18 M spermidine·Cl, 50 mM DTT, 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. Supernatants were decanted and pooled (about 1200 ml/900 to 1200 g cell suspension). Fraction I represents five such preparations pooled after the next step. *II, Ammonium Sulfate:* Solid ammonium sulfate (220 g/liter) was added slowly to Fraction I. The mixture was stirred 20 min and then centrifuged 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 M 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 (equivalent to 200 mM NaCl), applied to a 1250-ml DE 52 column (9 × 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 BioRex 70 column (7.5 × 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 eluate (Fig. 1*A*). Peak fractions were pooled and concentrated with ammonium sulfate (0.35 g/ml). Pellets, frozen in liquid nitrogen and stored at −70°C, were redissolved 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 × 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 + 1 M NaCl (Fig. 1*B*). 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 × 6.5 cm) equilibrated with 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. 1*C*). 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.[2]

Step	Total protein	Replication assay		ATPase assay		Ratio: ATPase / replication
		Total activity	Specific activity	Total activity	Specific activity	
	mg	*units* × 10^{-8}	*units* × 10^{-4}/*mg*	*units* × 10^{-8}	*units* × 10^{-4}/*mg*	
I Lysis	87,000	90	10	450	52	5.2
II Ammonium sulfate	21,000	78	37	260	120	3.3
IIIa DEAE-cellulose I	13,000	48	36	170	130	3.5
IIIb Bio-Rex 70	580	30	520	75	1300	2.5
IV DNA-cellulose	79	18	2300	54	6800	3.0
V DEAE-cellulose II	30	14	4700	29	9700	2.1

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 *rep*3 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 *ilv*$^+$ and *cya*$^+$ markers, complemented the chromosomal *rep* deficiency. The plating efficiency of G4 phage on HF4704 *rep*3/F$^+$/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 MV12/F$^+$ 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 ColE1 replication and also lyses well. The strain JFS19, so derived, which is *rep*$^+$ on the chromosome as well, was shown to contain the plasmid by transfer to HF4704 *rep*3, 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

[2] K. Arai and A. Kornberg, personal communication.

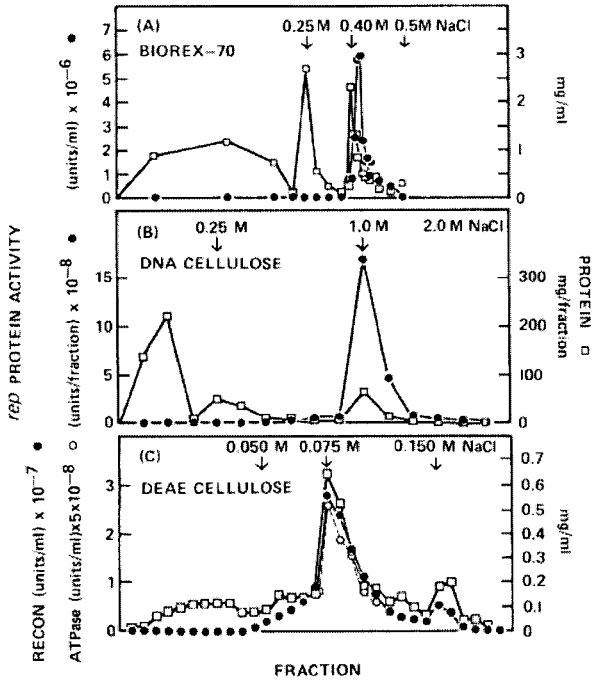

FIG. 1. Successive steps in the chromatographic purification of *rep* protein. *A*, Bio-Rex 70. The abscissa is linear with respect to volume; each point represents a single fraction. Fractions were of the following volumes: [1] 3900 ml; [2] 3450 ml; [3] 1250 ml; [4 to 8 and 18 to 20] 625 ml; and [9 to 17] 125 ml. The position of the symbols (*rep* protein activity (●——●); protein (■——■)) indicates the center of the fraction. *Rep* protein activity was determined by Assay B ("Materials and Methods"). The fractions in which each of the salt fronts appear are marked with arrows and the appropriate NaCl concentration of the step. *B*, DNA-cellulose. The abscissa is not linear with respect to volume. Fraction volumes were as follows: [1] 225 ml; [2] 300 ml; [3 to 15] 45 ml. The position of the symbols indicates the centers of the fractions and the ordinate is indicative of the total quantity of activity and protein in the fractions. The assays and symbols are as in *A*. *C*, DEAE-cellulose. The abscissa is linear with respect to volume. All fractions were 15 ml. The assays and symbols are as in *A* with the addition of *rep* ATPase activity (○- - -○) determined by Assay C ("Materials and Methods").

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. 1*c*), 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 ϕX 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%

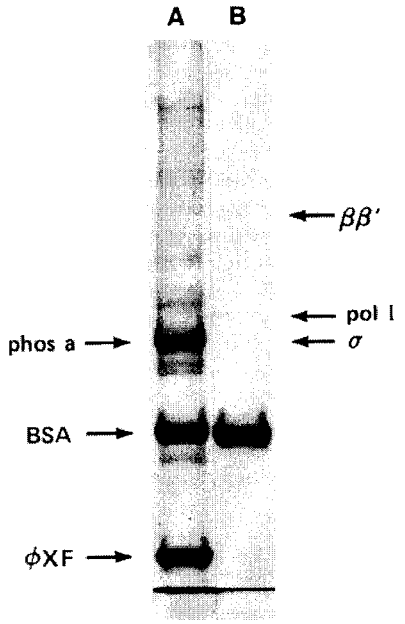

FIG. 2. SDS-polyacrylamide gel electrophoresis of *rep* protein. The slab gel contained 6% acrylamide, 0.16% bisacrylamide, 0.1% SDS, with a 3% stacking gel containing 0.08% bisacrylamide, and 0.1% SDS. The gel was run in a Tris·glycine system containing 0.1% SDS in the upper tray buffer. Samples were treated with 1% (v/v) 2-mercaptoethanol in Tris·Cl, pH 6.8, at room temperature for 10 min and at 100°C for 2 min before loading on the gel. *Track A* contained 5 μg each of the following markers: phosphorylase *a* (*phos a*) (92,500 daltons); albumin (*BSA*) (66,000 daltons); and ϕX F protein (46,000 daltons). *Track B* contained 5 μg of Fraction V *rep* protein. Other markers run in parallel tracks not shown were *E. coli* RNA polymerase BB' and σ subunits (155,000, 135,000, and 90,000 daltons, respectively) and DNA polymerase I (*pol I*) (109,000 daltons).

TABLE III

Amino acid composition of rep protein

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	moles/65,000 g
Alanine	46.2
Arginine	41.9
Aspartic acid, asparagine	54.1
Half-cystine	3.5
Glutamic acid, glutamine	80.7
Glycine	31.0
Histidine	11.8
Isoleucine	26.2
Leucine	72.0
Lysine	31.4
Methionine	13.5
Phenylalanine	24.9
Proline	17.9
Serine	29.7
Threonine	31.4
Tryptophan	5.8
Tyrosine	18.8
Valine	25.7

inhibition of the uncoupled ATPase activity observed using Assay C (based on ATP hydrolysis by *rep* in the presence of ϕX SS DNA effector). This result provides yet another example of the special characteristics of *rep* protein at the replication fork when compared with the uncoupled ATPase reaction using single-stranded DNA effector (see succeeding paper (9)).

Amino Acid Analysis of rep Protein – Samples of *rep* protein were subjected to amino acid analysis (Table III). The predominant residues in the protein are glutamic and aspartic acids (including glutamine and asparagine) and leucine. No residue appears to be absent or present in a single copy in the polypeptide, nor are any other striking features apparent. A comparable sample of *rep* protein was also analyzed by Dr. Marian Koshland at the University of California, Berkeley.[3] The two analyses were in good general agreement, and both included indications of possible modified half-cystine residues present. Such residues have not as yet been positively identified.

DISCUSSION

To provide a better source of *rep* activity for purification of the enzyme, a bacterial strain was constructed which contained several copies of *rep* gene in ColE1/*E. coli* hybrid plasmids. This strain provided a 7- to 10-fold increase in production of *rep* protein compared to the standard strain. Its use, coupled with improved yields from the early steps of the purification procedure, have enabled us to prepare pure *rep* enzyme in quantities sufficient for future physical studies.

The *rep* protein has a polypeptide molecular weight of 65,000 when purified from either the overproducing strain (JFS19) or from the standard strain (HMS83) used routinely in this laboratory. Sedimentation of *rep* in a glycerol gradient (20) yielded an apparent native molecular weight of 85,000 to 90,000. This value while higher than expected for a monomer, falls short of the value for a globular dimer. Cross-linking studies to determine whether *rep* protein is oligomeric have thus far been equivocal.

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 ColE1/*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 of ϕX SS to RF (28), a 180,000-dalton DNA unwinding enzyme I (29, 30), *rec* B-C nuclease (31), type I restriction endonucleases (*e.g.* EcoK12, EcoB, EcoP1) (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 *cistron* 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 unzippering 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

[3] M. Koshland, personal communication.

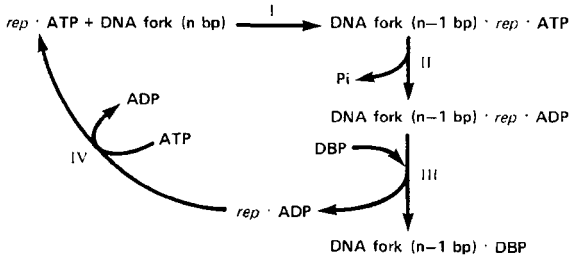

FIG. 3.

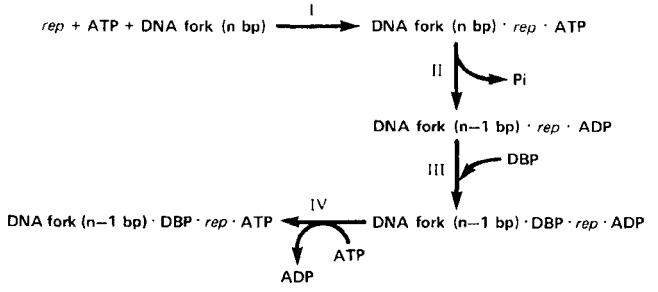

FIG. 4.

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.

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