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Ten Proteins Required for Conversion of φX174 Single-stranded DNA to Duplex Form *in Vitro*

RESOLUTION AND RECONSTITUTION*

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SUMMARY

Protein requirements for conversion of φX174 single-stranded DNA to a double-stranded replicative form with a small gap (RF II) have been determined by resolution and reconstitution of the multienzyme system from extracts of gently lysed *Escherichia coli*. Assays depended on: (*a*) complementation of extracts of thermosensitive mutants and (*b*) fractionation of extracts of wild type cells to divide essential components into groups, each of which was further resolved. These procedures have yielded eight proteins: *dnaB* protein, *dnaC* protein, proteins i and n (two novel proteins without a defined genetic locus), *dnaG* protein, DNA polymerase III holoenzyme (polymerase III* and copolymerase III*), and DNA unwinding protein; purification procedures for the first four are presented here. (Closure of RF II requires as with phage M13, DNA polymerase I and ligase.)

Soluble extracts of gently lysed *Escherichia coli* convert φX174 single-stranded DNA to the double-stranded replicative form (1). Enzymes involved in this reaction appear to participate in host chromosome replication. Conditionally lethal mutants of *E. coli*, defective in initiation at the chromosome origin (*dnaA*, *dnaC*) and ongoing DNA replication (*dnaB*, *dnaE*, and *dnaG*) all show *in vitro* defects in φX174 RF[1] formation (2, 3). Proteins corresponding to these *dna* genes may be assayed and purified using complementation of extracts prepared from mutant cells. Partially resolved enzyme fractions have been added to deficient extracts and assayed at an elevated temperature at which the mutant protein is inactive. The *dnaB*, *dnaC*, *dnaE*, and *dnaG* gene products have been purified in this manner (4–7). However, these four proteins were insufficient for RF synthesis from single-stranded DNA.

In another approach extracts from wild type cells are subjected to fractionation. A procedure is adopted which generates two fractions, each of which is required; each fraction is then subdivided further. This approach together with the complementation assay has been employed for complete resolution of the components involved in the φX174 reaction (8, 9). This report presents the strategy of the fractionation pattern, and the assays and purification procedures developed for four of the essential proteins. Information about the size, properties, abundance, and possible functions of these proteins are suggested from these studies.

EXPERIMENTAL PROCEDURE

Materials

Materials were from sources previously described (1, 2). Freshly dissolved calf thymus DNA (Calbiochem) was denatured and attached to cellulose by the procedure of Alberts (10) dried at 37° and stored as a powder at −20°.

Buffer A is 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 20% glycerol (v/v), 1 mM EDTA, and 1 mM dithiothreitol. Buffer B is 50 mM imidazole-HCl (pH 7.0), 20% glycerol, 1 mM EDTA, and 1 mM dithiothreitol. Buffer C is 50 mM Tris-HCl (pH 7.5), 30% glycerol, 1 mM EDTA, and 10 mM dithiothreitol. Buffer D is 50 mM Tris-HCl (pH 7.5), 20% glycerol, and 1 mM EDTA. Buffer E is 20 mM potassium phosphate (pH 6.5), 20% glycerol, and 1 mM EDTA. Tris-HCl and imidazole-HCl stock solutions (2 M) were prepared at room temperature and diluted as required.

DNA unwinding protein was Fraction 3b (0.38 mg/ml (11)); *dnaG* protein (Fraction V, 0.04 mg/ml)[2] was prepared according to Bouché *et al.*[2] DNA polymerase III holoenzyme was purified as before (12) except that Bio-Gel A-0.5m (200 to 400 mesh) replaced Bio-Gel A-5m. Fraction IV was precipitated with an equal volume of saturated ammonium sulfate (neutralized with ammonium hydroxide). After 30 min at 0° the precipitate was centrifuged at 31,500 × *g* for 10 min and the supernatant was discarded. Ammonium sulfate precipitates stored at 0° were stable for at least 2 months; when redissolved in Buffer A (containing 10 mM dithiothreitol) they retained 50% of their activity after 1 week at 0°.

Escherichia coli PC 79 (F^-, his^-, str^R, malA, xyl^-, mtl^-, thi^-, pol A1, sup^-, *dnaD7* (now referred to as *dnaC*)) was provided by Dr. P. Carl (University of Illinois, Urbana). *E. coli* BT1029 (*dnaB*, thy^-, pol A1, endo I^-) was provided by Dr. H. Hoffmann-Berling (Heidelberg). *E. coli* H560 was grown and soluble extracts were prepared as previously described (1, 12) except that the 37° heating step was performed with 35-ml aliquots in polyethylene centrifuge

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[1] The abbreviations used are: RF, circular double-stranded DNA of replicative form; RFI, covalently closed RF, RF II, RF with a discontinuity in one strand; NEM, *N*-ethylmaleimide.

[2] J.-P. Bouché, K. Zechel, and A. Kornberg (1975) *J. Biol. Chem.* **250**, in press

tubes for 2 min followed by centrifugation at 48,000 × *g* for 30 min at 0°. *E. coli* HMS-83, provided by Dr. R. L. McMacken, of this department, was grown as *E. coli* H560 (1).

Methods

General Procedures

Ammonium Sulfate Precipitation—Solid ammonium sulfate was added at 0° over a 10-min interval, the solutions were stirred for another 20 min and centrifuged for 10 min at 23,000 × *g*. Solutions for extracting ammonium sulfate precipitates contained varying amounts of ammonium sulfate added to Buffer A (*e.g.* solution AS 0.20 was made up by adding 20 g of ammonium sulfate to 100 ml of Buffer A). A glass homogenizer was used manually for these extractions.

Assay of dnaC Protein

Preparation of Mutant Enzyme Fraction—E. coli PC79 was grown at 30° in Hershey broth, harvested, and resuspended as described before for *E. coli* H560 (1). The soluble extract (Fraction I) was prepared as before (1, 12) except that spermidine was omitted and lysis was for 1 hour at 0°. Fraction I was adjusted to 0.2 M NaCl and applied to a column of DEAE-cellulose (equilibrated in Buffer D + 0.2 M NaCl) ⅕th the volume of Fraction I. The flow-through was collected and solid ammonium sulfate was added to 40% saturation (0.242 g added/ml). The solution was stirred for 30 min at 0°, and then centrifuged at 39,000 × *g* for 10 min. The pellet (Fraction II) was taken up in Buffer D, 1/20th the volume of Fraction I (about 10 mg of protein/ml) and dialyzed against 100 volumes of Buffer D + 0.05 M NaCl for 4 hours. The dialyzed fraction divided into aliquots was stored in liquid nitrogen and remained stable for at least 3 months.

Assay—Assays were performed in 25 μl at 30° for 20 min. Components were added in the following order at 0°: 5 to 10 μl of dilution buffer (10% sucrose, 50 mM Tris-HCl (pH 7.5), 20 mM dithiothreitol, 0.2 mg/ml of bovine serum albumin, and 50 mM NaCl), 40 μg (4 μl) of mutant Fraction II, 1 to 6 μl of the fraction to be assayed, and 10 μl of a reaction mixture containing 0.45 nmol of [α-^{32}P]dCTP (200 to 600 cpm/pmol), 1.25 nmol each of dATP, dGTP, and TTP, 125 nmol of $MgCl_2$, 100 nmol of spermidine-HCl, 20 nmol of ATP, 2.5 nmols each of GTP, UTP, and CTP, 0.33 nmol of φX174 DNA, and 100 ng of rifampicin. DNA synthesis was measured by incorporation of the labeled deoxynucleotide into an acid-insoluble form. One unit is defined as 1 pmol of total deoxynucleotide incorporated in 1 min; the value for dCMP incorporation was multiplied by 4.

Preparation of DNA-cellulose Binding and Nonbinding Fractions

E. coli H560 (150 g of cell paste in 750 ml of 10% sucrose and 50 mM Tris-HCl (pH 7.5)) was lysed and centrifuged to yield Fraction I (500 ml) (Table I) (1, 2). Ammonium sulfate (0.226 g/ml) was added and the precipitate was collected as described above. The precipitate was resuspended in 50 ml of AS 0.24 and the insoluble proteins were collected. This procedure was repeated with 10 ml of AS 0.20. The precipitate was resuspended in Buffer D to a final volume of 11 ml and applied to a Sephadex G-25 column (170 ml, 3-cm diameter) equilibrated in and eluted with Buffer D. The void volume fractions of column buffer conductivity (1.8 mmho) were pooled (42 ml, Fraction II) and solid NaCl was added to a final concentration of 0.2 M. This fraction was then applied to a DEAE-cellulose (DE52) column (10 ml, 2-cm diameter) equilibrated in Buffer D + 0.20 M NaCl, followed by a 10-ml wash with the same buffer. Fractions containing the bulk of the protein were pooled (50 ml), ammonium sulfate was added (0.272 g/ml), and the precipitate was collected and resuspended in 5 ml of Buffer B. This fraction was then applied to a Sephadex G-25 column (45 ml, 2-cm diameter) equilibrated in and eluted with Buffer B.

The void volume fractions of column buffer conductivity (1.7 mmho) were pooled (15 ml, Fraction III) and applied to a DNA-cellulose column (12 ml, 3.5-cm diameter) also equilibrated in Buffer B. The column was then washed with 100 ml of Buffer B. Nonbinding (flow-through) fractions (5 ml) were collected and assayed as described below. The DNA-cellulose binding proteins were eluted with 40 ml of Buffer B + 2 M NaCl, precipitated with ammonium sulfate (0.226 g/ml), resuspended in 2.5 ml of Buffer B, dialyzed against 500 ml of Buffer D + 0.2 M NaCl, and stored in aliquots in liquid nitrogen for at least 6 months with no loss of activity. Ammonium sulfate precipitates of Fractions II or III may be stored at −20° for several days and subsequently carried through the remaining steps.

Resolution of DNA-cellulose Nonbinding Proteins

The nonbinding fractions from the above preparation were pooled (35 ml), precipitated with ammonium sulfate (0.272 g/ml), collected, and resuspended in 1.5 ml of Buffer A. Part of the sample (1.2 ml) was applied to a Sephadex G-150 column (42 ml, 2-cm diameter) equilibrated in Buffer A and eluted by this buffer. Two essential proteins were separated (Fig. 1); one was in the void volume (*dna*B protein) and the other was included at K_{av} = 0.25 (protein i). Peak fractions of each were pooled and aliquots stored in liquid nitrogen. Quantitative determination of the preparation through this stage is presented in Table I.

Assay of DNA Binding and Nonbinding Fractions

Activity of the binding fraction was determined by assaying varying amounts in the presence of *dna*C protein and the nonbinding fraction. Assays were in 25 μl at 30° for 20 min. Components were mixed as follows: 10 μl of dilution fraction, 2 μl of nonbinding fraction, 0.2 to 2 μl (1 to 10 μg) of binding fraction, 2 to 5 μg of Fraction III *dna*C protein, and 10 μl of the reaction mixture described for assay of the *dna*C protein. DNA synthesis was measured as above. When the resolved nonbinding fractions were purified and became available, protein i (Fraction V, 4 to 40 ng/assay) and *dna*B protein (Fraction V, 20 ng/assay) were used (see "Results").

Assay for Protein n, NEM-sensitive DNA-cellulose Binding Protein

The binding fraction contains two essential components which are inactivated by the sulfhydryl-blocking agent, NEM: DNA polymerase III holoenzyme and another component, called protein n. The binding fraction was treated with 10 mM NEM (freshly dissolved in water) at 30° for 15 min and excess NEM was removed with 20 mM dithiothreitol at 0°. Assays for protein n were mixed as follows: 10 μl of dilution buffer, 0.1 to 2 μl of the fraction to be assayed, 5 μl of a mixture of NEM-treated binding fraction (1 to 10 μg), protein i (Fraction V, 40 ng), *dna*B protein (Fraction V, 20 ng), DNA polymerase III holoenzyme (Fraction IV, 0.33 μg), and *dna*C protein (Fraction III, 2 to 5 μg), followed by 10 μl of the reaction mixture used for assay of the *dna*C protein. Alternatively, purified DNA unwinding protein (700 ng) plus *dna*G protein (20 ng) can replace the NEM-treated binding fraction.

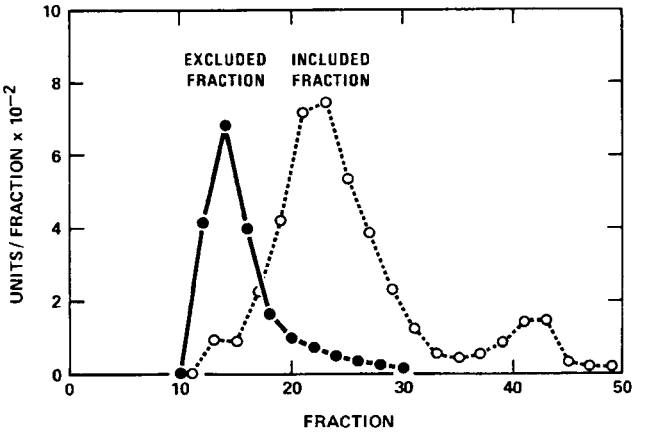

FIG. 1. Sephadex G-150 chromatography of the DNA-cellulose nonbinding fraction. The DNA-cellulose flow-through fraction (see "Methods") (1.2 ml, 13 mg, 470 units/mg) was applied to a Sephadex G-150 column (42 ml, 2-cm diameter) equilibrated in and eluted with Buffer A. Fractions of 1.25 ml were collected at a flow rate of 20 ml/hour. Aliquots (2 μl) of the void volume protein combined with portions (2 μl) of the column fractions were used to assay for the included fraction (see "Methods"). The excluded fraction was then assayed by combining 2-μl aliquots of the included peak with 2-μl portions of the void volume region.

TABLE I

Resolution of DNA-cellulose binding fractions, dnaB protein, and protein i

Assays were performed as under "Methods." Activity was measured with 2- to 4-μg additions of *dna*C protein (Fraction III). Stage II was assayed with *dna*C protein and an excess of binding or nonbinding fractions. Stage III was assayed with *dna*C protein, DNA-cellulose binding fraction, and either an excess of Sephadex G-150 excluded fraction or included fraction to permit titration of the component to be assayed.

Stages in fractionation	Total		
	Protein	Units	Specific activity
	mg/ml	$\times 10^{-3}$	*units/mg*
Extract	19.7		
Stage I			
Ammonium sulfate	2.7	90	790
DEAE-cellulose	6.1	124	1,360
Stage II			
DNA-cellulose binding fraction	5.3	121	9,100
Nonbinding fraction	11.7	119	6,800
Stage III			
Sephadex G-150 excluded fraction (*dna*B protein)	1.5	8.6	2,300
Included fraction (protein i)	0.6	40.2	13,400

Glycerol Gradient Sedimentations

These were performed in an SW 56 rotor of the Beckman L2-65B ultracentrifuge. Fractions were collected through a puncture at the bottom of the tube. Hemoglobin and myoglobin markers were located by measuring A_{420}.

RESULTS

Strategy of Fractionation

The fractionation pattern of an extract of wild type cells can be divided into five stages (Table II, Fig. 2).

Separation of dnaC Protein by Ammonium Sulfate—Fraction II (see "Methods" for preparation of DNA-cellulose binding and nonbinding fraction) was deficient in *dna*C protein (Table II, Stage I). The latter could be assayed with the *dna*C-depleted ammonium sulfate fraction from wild type cells or from *dna*C mutant cells (Table II, Footnote *a*).

Separation of DNA-cellulose Binding and Nonbinding Proteins—Fraction II (depleted of *dna*C protein) was applied to a column of DNA-cellulose and the nonbinding (flow-through) and binding fractions were collected. At this stage (Table II, Stage II), assays required *dna*C protein and both the DNA-cellulose binding and nonbinding fractions.

Resolution of DNA-cellulose Nonbinding Proteins on Sephadex G-150—Filtration resolved this fraction into excluded and included activities (Fig. 1). Both were needed in addition to the DNA-cellulose binding proteins and *dna*C protein (Table I; Table II, Stage III). The excluded activity was identified as the *dna*B gene product by further purification (see below) and complementation assays (Table III); the included activity, called protein i, also appeared to be a single component upon purification (see below), but no genetic locus has yet been found for it.

NEM-sensitive DNA-cellulose Binding Proteins—Reconstitution at Stage III (Table II) was inactive when the DNA-cellulose binding fractions were treated with NEM (Table II, Stage IV). The NEM-sensitive components could be resolved by Sephadex G-150 filtration into excluded and included fractions. The excluded fraction was identified as DNA polymerase III holoenzyme (12). The other NEM-sensitive activity, called protein n, could then be assayed upon the addition of NEM-treated DNA-cellulose binding proteins, DNA polymerase III holoenzyme, DNA-cellulose nonbinding proteins, and *dna*C protein. Protein n has been purified to near homogeneity (see below) but has not been identified with any of the known genetic loci.

NEM-resistant DNA-cellulose Binding Proteins—Complete reconstitution was achieved by replacing the NEM-treated DNA-cellulose binding fraction by the *E. coli* DNA unwinding protein (11) and *dna*G protein (Table II, Stage V). Purification of

TABLE II

Stages in reconstitution

Assays were performed as described under "Methods." Where indicated, reactions contained 2 to 5 μg of *dna*C protein (Fraction III), 20 μg of Fraction II (depleted of *dna*C protein), 10 μg of DNA-cellulose binding proteins, 10 μg of DNA-cellulose nonbinding proteins, 40 ng of protein i (Fraction V), 20 ng of *dna*B protein (Fraction IV), 10 μg of NEM-treated DNA-cellulose binding proteins, 0.3 μg of DNA polymerase III holoenzyme (Fraction IV), 0.13 μg of protein n (Fraction V), 40 ng of *dna*G protein (Fraction V), and 0.73 μg of DNA unwinding protein (Fraction 3b).

Component omitted	Stages				
	I[a]	II	III	IV	V
	pmol DNA synthesis				
None (complete mixture)	88	52	47	37	120
*dna*C protein	4.0	9.1	0.4	6.4	7.9
Fraction II (depleted of *dna*C protein)	2.0				
DNA-cellulose binding proteins		2.0	1.1		
DNA-cellulose nonbinding proteins		1.9			
Protein i			6.7	8.7	13.9
*dna*B protein			4.0	7.6	24.9
NEM-treated DNA-cellulose binding proteins				6.7	
DNA polymerase III holoenzyme				1.8	2.2
Protein n				6.6	5.4
*dna*G protein					8.0
DNA unwinding protein					1.9

[a] A complementation assay of the *dna*C protein (Fraction III) gave a value of 60 pmol with the *dna*C mutant Fraction II (40 μg), but only 4 pmol without it; the value for Fraction II by itself was 2.0 pmol.

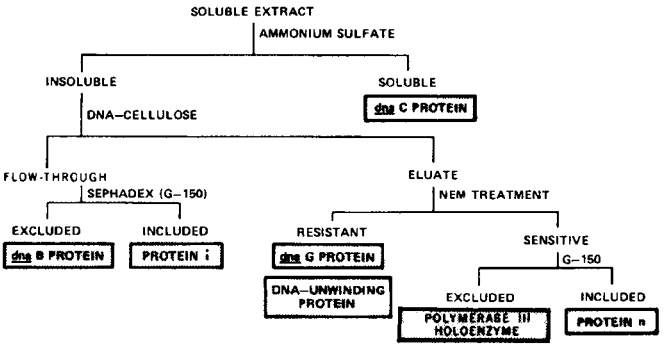

FIG. 2. Scheme for resolution of the φX174 replicative enzymes.

TABLE III

dnaB Complementation assay

Escherichia coli BT1029 was grown and soluble extracts were prepared as described for *dna*C mutant enzyme fraction. Ammonium sulfate was added to Fraction I (0.240 g/ml) at 0°. The suspension was centrifuged at 10,000 × *g* for 20 min. The pellet was dissolved in 1/80 volume of Buffer A without NaCl. Assays were performed as described for *dna*C complementation. Additions of *dna*C protein were 9 units of Fraction III.

Protein added to *dna*B mutant fraction	*dna*C protein	
	None	Present
	pmol DNA synthesis	
None	8.0	11.8
*dna*B protein	13.5	54.6
DNA unwinding protein	12.2	11.4
Protein i	11.3	17.3
Protein n	12.3	12.3
*dna*G protein	12.2	9.0
DNA polymerase III holoenzyme	12.7	9.4

these proteins based on their role in the conversion of phage G4 single-stranded DNA to RF are presented elsewhere (11).[2]

The product at Stage V was RF II with a nearly full length linear synthetic strand (8).

The procedures described above and outlined in Fig. 2 were guides to the existence and general properties of the several proteins and provided assays for them. The endogenous (over-all) replication activity of the extract was 800 to 1000 units/g of cell paste. On this basis, the activity of the individual components, expressed as units/g of cell paste, was: *dna*B protein, 7000; *dna*C protein, 3000; *dna*G protein, 8000; DNA polymerase III holoenzyme, 5000; protein n, 1400; protein i, 1100 units. In recent experiments with protein i activity levels of 5000 units/g of cell paste have been observed. Thus, each of the activities was present at a level equal to or greater than the endogenous activity. Independent purification procedures had to be developed to obtain each protein in optimal yields. The purification procedures for *dna*C protein, protein i, *dna*B protein, and protein n are given below.

Purification of dnaC Protein

This protein is the activity most readily identified as specific for φX174 and also the most labile. Although *dna*C activity can be isolated from *E. coli* H560, only 165 units/g of cell paste were recovered in extracts compared to 2600 to 3000 units/g for *E. coli* HMS-83. Beyond the enrichment of about 70-fold (Table IV) further attempts at purification have been frustrated by loss of activity.

Glycerol gradient sedimentation of *dna*C protein (Fig. 3) gave an S value of 2.0 corresponding to a molecular weight of about 20,000.

Purification of dnaB Protein

The purification of *dna*B protein is summarized in Table V. This protein was initially identified as the excluded factor on Sephadex G-150 gel filtration and subsequently shown to complement a crude fraction prepared from a *dna*B temperature-sensitive mutant (Table III). In order to complement this mutant it was necessary to add *dna*C protein in addition to *dna*B protein. Similar pleiotropic requirements seen with other mutant extracts

TABLE IV

Purification of dnaC protein

Escherichia coli HMS83 (335 ml, 65 g) prepared as for H560 (see "Methods") was thawed at 10° and diluted with 150 ml of 0.05 M Tris-HCl (pH 7.5)/10% sucrose. To the suspension was added 1.2 ml of 0.4 M dithiothreitol, 18.5 ml of 4 M KCl, 4.8 ml of lysozyme (20 mg/ml), 4.8 ml of 10% Brij 58, 19.5 ml of 0.5 M EDTA, and 9.7 ml of 1 M spermidine-HCl. The pH was adjusted to 8.5 with 1.5 g of solid Tris base. The suspension was incubated for 25 min at 4°, then centrifuged at 48,000 × *g* for 30 min to produce Fraction I (415 ml). Ammonium sulfate (0.240 g/ml) was added; the precipitate collected as described under "Methods" was resuspended in 33.5 ml of AS 0.24. The undissolved material was collected and dissolved in Buffer B containing 0.2 M KCl (Fraction II, 16 ml). Fraction II (5.5 ml) was dialyzed against two 1-liter changes of Buffer B plus 0.2 M KCl for a total of 2.5 hours. The sample was diluted 1 to 5 in successive 1-ml portions in Buffer B and applied to a phosphocellulose column (45 ml; 2.5-cm diameter) equilibrated in Buffer B, the column was washed with 45 ml of Buffer B and *dna*C activity was eluted with a linear KCl gradient (250 ml, 0.05 to 0.4 M in Buffer B). Pooled fractions (40 ml) precipitated with ammonium sulfate (0.39 g/ml) were dissolved in 4 ml of Buffer B containing 0.2 M KCl (Fraction III) and stored in aliquots in liquid nitrogen where it was stable for at least 3 months. Fraction II may be stored at −20°, but is unstable at 0°.

Fraction	Total units	Protein	Specific activity	Yield	Purification
	× 10^{-3}	*mg/ml*	*units/mg*	%	
I. Extract	284	17	40		
II. Ammonium sulfate [a]	214	78	170	75	4.3
III. Phosphocellulose[b]	156	4.5	2650	55	70

[a] Calculation of yield and purification was based on the *dna*C activity recovered in all of the ammonium sulfate fractions, assays in Fraction I were not reliable.

[b] This step was carried out in a portion of Fraction II (5.5 ml) but is calculated for the entire preparation.

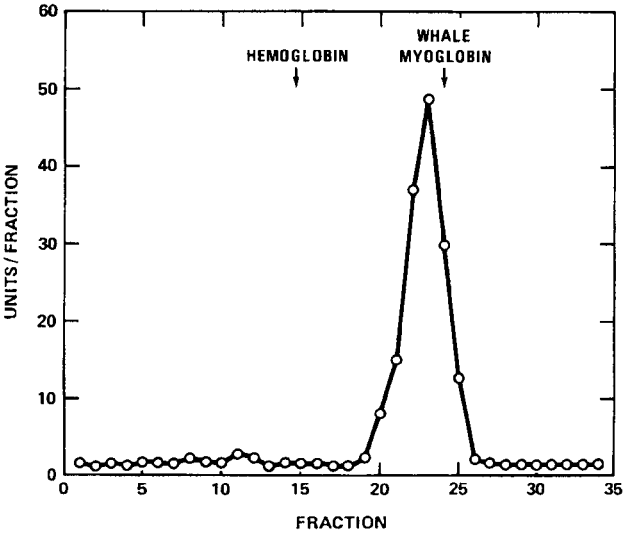

FIG. 3. Glycerol gradient sedimentation of *dna*C protein. *dna*C protein (75 μg, 300 units) was applied to a 3.8-ml 10 to 25% glycerol gradient in 0.05 M imidazole-HCl (pH 7.0)/0.2 M KCl/1 mM EDTA/1 mM dithiothreitol. Centrifugation was at 55,000 rpm for 24 hours at 0°. Thirty-four fractions were collected, assayed, and marker positions were determined as described under "Methods."

TABLE V

Purification of dnaB protein

Escherichia coli H560 (1.5 liters, 300 g of frozen cells) was lysed to yield 1180 ml of Fraction I. Ammonium sulfate was added (0.226 g/ml); the precipitate was collected and resuspended in 118 ml of AS 0.20; the pellets were treated successively with 24-ml aliquots of AS 0.16, AS 0.15, and AS 0.14. The supernatant fluids from these last three washes were combined and the *dna*B activity reprecipitated with ammonium sulfate (0.1 g/ml). The precipitate was collected and resuspended in Buffer D containing 0.25 M NaCl (Fraction II, 10 ml). The sample was applied to a column of Bio-Gel A-1.5m (200 to 400 mesh, 475 ml, 3.8-cm diameter) equilibrated in and eluted with Buffer D containing 0.25 M NaCl. Fractions with *dna*B activity (100 ml), eluted at a K_{av} of 0.36 to 0.64, were treated with ammonium sulfate (0.24 g/ml); the precipitate was dissolved in 3 ml of Buffer D containing 0.05 M NaCl (Fraction III, 3.2 ml). The sample was applied to a column of Sephadex G-25 (40 ml, 2-cm diameter) equilibrated in Buffer D containing 0.05 M KCl and the void volume fractions were pooled (10 ml) and applied to a column of DEAE-cellulose (20 ml, 2-cm diameter) equilibrated in Buffer D (0.05 M NaCl). The column was then washed with 10 ml of the same buffer followed by 30 ml of Buffer D containing 0.15 M NaCl; *dna*B activity was eluted with a linear NaCl gradient (120 ml, 0.15 to 0.40 M in Buffer D) at about 0.35 M Cl^- and concentrated on DEAE-cellulose. Aliquots (2.5 ml) of the pooled activity (32 ml) were each diluted with 3 volumes of 20% glycerol/1 mM EDTA and applied sequentially to a column of DEAE-cellulose (2 ml, 1-cm diameter) equilibrated in Buffer D. The activity was then step eluted with 10 ml of Buffer D + 0.40 M NaCl and 1-ml fractions were collected. The *dna*B activity was pooled (Fraction IV, 2.8 ml) and dialyzed against three 500-ml changes of Buffer E for a total of 5 hours. The sample was applied to a column of phosphocellulose (10 ml, 1-cm diameter) equilibrated in Buffer E. The column was then washed with 20 ml of Buffer E and *dna*B activity eluted with a linear KCl gradient (30 ml, 0 to 0.40 M KCl in Buffer E) at about 0.3 M Cl^-. The pooled activity (Fraction V, 6.2 ml) was stored in aliquots in liquid nitrogen where it was stable for at least 3 months.

Fraction II may be stored at −20°. The activity is not stable at low ionic strength (<0.1 M Cl^-) at 0° for more than 1 day.

Fraction	Total units	Protein	Specific activity	Yield	Purification
	× 10^{-3}	mg/ml	units/mg × 10^{-3}	%	
I. Extract	2000	20	0.087		
II. Ammonium sulfate[a]	1720	13.2	13	81	150
III. Bio-Gel A-1.5m	555	8.8	25	34	290
IV. DEAE-cellulose	295	0.3	350	14	4020
V. Phosphocellulose	73	0.02	590	4	6780

[a] Calculation of yield and purification was based on the activity recovered in all of the ammonium sulfate fractions; assays in Fraction I were not reliable.

have complicated the use of this approach to purify the deficient protein.

Preliminary characterization of *dna*B protein indicates a native molecular weight of about 250,000 in agreement with the published value (4). The function of *dna*B protein has not yet been elucidated.

Purification of Protein i

Protein i was purified 30,000-fold by the procedure summarized in Table VI. Sodium dodecyl sulfate gel electrophoresis indicated a single major band which migrated slightly slower than DNA unwinding protein and thus had a monomer molecular weight of about 20,000.[3] Glycerol gradient sedimentation of protein i (Fig. 4) gave an S value of 3.0 corresponding to a molecular weight of about 35,000 to 40,000 indicating protein i is either an asymmetric monomer or a dimer. From the data of Table VI we estimate about 100 to 150 molecules of protein i per cell. No function for protein i has yet been determined. Based on similarities in the purification procedure and physical properties it appears that protein i corresponds to the Factor X recently reported by Wickner and Hurwitz (9).

TABLE VI

Purification of protein i

Fraction I (2130 ml) prepared from a lysate of *Escherichia coli* H560 (3.0 liters, 600 g) was treated with ammonium sulfate as described for *dna*B protein (Table V) except that 213 ml of AS 0.30 and 43 ml of the AS 0.16, AS 0.15, and AS 0.14 were used. The final precipitate (AS 0.14 pellet) which contained the bulk of protein i activity (as well as DNA polymerase III holoenzyme (12)) was resuspended in 25 ml of Buffer A (Fraction II, 30 ml), clarified, and applied to a column of Sephadex G-25 (250 ml, 3-cm diameter) equilibrated in and eluted with Buffer C. The Sephadex G-25 void volume fractions of conductivity equivalent to Buffer C were pooled and applied to a column of DEAE-cellulose (50 ml, 2-cm diameter) equilibrated in Buffer C. Protein i activity was eluted with a linear NaCl gradient (500 ml, 0 to 0.3 M NaCl in Buffer C) at about 0.15 M Cl^- (Fraction III, 50 ml), concentrated with ammonium sulfate (0.24 g/ml) and resuspended in 5 ml of Buffer D. This sample was applied to a column of Sephadex G-150 (250 ml, 2.5-cm diameter) equilibrated with Buffer D. Protein i activity was eluted at K_{av} = 0.25 (Fraction IV, 30 ml). This fraction was concentrated on a column of DEAE-cellulose (2.5 ml, 1.2-cm diameter). After equilibration in Buffer D, protein i activity was step eluted with 10 ml of Buffer D containing 0.2 M NaCl; protein i (5 ml) was dialyzed against two 500-ml changes of Buffer E for a total of 5 hours and immediately applied to a column of phosphocellulose (5 ml, 1-cm diameter) equilibrated in Buffer E. The column was washed with 4 ml of Buffer E and protein i activity eluted with a linear KCl gradient (50 ml, 0 to 0.4 M KCl in Buffer E) at about 0.2 M Cl^-. The pooled activity (Fraction V, 5 ml) was stored in aliquots in liquid nitrogen where it was stable for at least 6 months.

Fraction II may be stored at −20°, but once dissolved should be chromatographed on DEAE-cellulose immediately.

Fraction	Total units	Protein	Specific activity	Yield	Purification
	× 10^{-3}	mg/ml	units/mg × 10^{-3}	%	
I. Extract	690	25	0.013		
II. Ammonium sulfate[a]	420	1.9	7.3	60	590
III. DEAE-cellulose	138	0.23	12	21	980
IV. Sephadex G-150	113	0.14	27	17	2,180
V. Phosphocellulose	80	0.043	370	12	29,600

[a] Calculation of yield and purification was based on the activity recovered in all of the ammonium sulfate fractions; assays in Fraction I were not reliable.

Purification of Protein n

Protein n was purified about 2,000-fold by the procedure in Table VII. Sodium dodecyl sulfate gel electrophoresis indicated a single major band at 82,000 daltons and minor bands at 25,000 and 50,000 daltons. The activity appeared to be associated with the major band. Fraction VI was estimated to be about 80% pure by Coomassie blue staining. From the data of Table VII about

[3] R. McMacken and A. Kornberg, unpublished work.

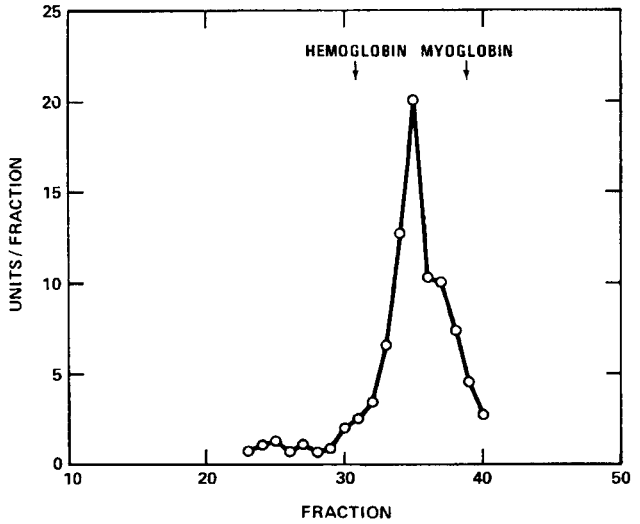

FIG 4. Glycerol gradient sedimentation of protein i. Protein i (2.4 μg, 900 units) was applied to a 3.8-ml, 10 to 30% glycerol gradient in: 0.05 M Tris-HCl (pH 7.5)/0.20 M NaCl/1 mM EDTA. Centrifugation was at 55,000 rpm for 16 hours at 0°. Forty-three fractions were collected, assayed, and marker positions were determined as described under "Methods."

200 molecules of protein n are present per cell. Protein n aggregates at protein concentrations greater than 0.1 mg/ml and salt concentrations less than 0.25 M making estimation of the native molecular weight inaccurate. The protein binds specifically to single-stranded DNA dramatically altering the contour length.[4]

DISCUSSION

What seemed to be a simple operation of copying the small, single-stranded DNA circle of φX174 to form the duplex requires a multienzyme system. The many proteins which make up this system hold great interest because they appear to be the components which the uninfected *Escherichia coli* uses to initiate and sustain the replication of its own chromosome. Thus, the resolution and reconstitution of this system made possible by the simplicity of the phage DNA template has implications for understanding replication of duplex DNA of *E. coli*. In an analogous way the filamentous phage M13 has been a useful probe for another replicative system in *E. coli*, one which appears to be reserved for replication of extrachromosomal elements (13).

It simplifies discussion to consider three major stages in the synthesis of a DNA chain: initiation, elongation, and termination. Both the φX174 and M13 replication systems are similar in their requirement for an RNA-primer fragment synthesized in the initiation stage, for the DNA polymerase III holoenzyme and DNA unwinding protein to sustain the elongation stage, and for DNA polymerase I and ligase to carry out the termination stage (*e.g.* RF II conversion to RF I) (8). The two systems differ in the mechanism used for synthesis of the RNA primer. Initiation on M13 DNA depends on a form of the multisubunit RNA polymerase and so is sensitive to inhibition by rifampicin. The φX174 system, on the other hand, is resistant to rifampicin and depends on a novel RNA synthetic enzyme.[2]

Eight proteins have been resolved from extracts of *E. coli* that are needed for reconstituting the φX174 single strand conversion to RF II. Available information about their physical and functional properties, summarized in Table VIII, is incomplete in most instances. It seems clear that the polymerase III* and copolymerase III* (6) subunits of the DNA polymerase III holoenzyme are needed for DNA synthesis and that binding of the DNA template by the DNA unwinding protein is required both for initiation and elongation.

A strong clue to the function of the *dna*G protein has been furnished recently by its action in the conversion of the viral single-stranded DNA of G4, a phage related to φX174.[5] In this case, the RNA primer is synthesized by the *dna*G protein in the presence of DNA unwinding protein;[2] the other proteins needed

[4] J. H. Weiner and A. Kornberg, unpublished work.

[5] K. Zechel, J.-P. Bouché, and A. Kornberg (1975) *J. Biol. Chem.* **250**, in press.

TABLE VII

Purification of protein n

Escherichia coli H560 (700 ml, 140 g) was lysed to yield Fraction I (575 ml). Ammonium sulfate was added (0.226 g/ml) and the precipitate collected and resuspended in 58 ml of AS 0.24. The precipitate was collected and the procedure repeated with 11-ml aliquots of AS 0.20 and AS 0.18. The final precipitate was collected, dissolved in 8.5 ml of Buffer D containing 1 mM dithiothreitol, clarified and applied to a Sephadex G-25 column (150 ml, 3-cm diameter) equilibrated in and eluted with Buffer D (1 mM dithiothreitol).

To the pooled void volume fraction NaCl was added to a concentration of 0.2 M. Fraction II (34 ml) was applied to a DEAE-cellulose column (10 ml, 2-cm diameter) equilibrated in and eluted with the same buffer. The protein fractions in the flow-through were pooled, precipitated with ammonium sulfate (0.313 g/ml), and the precipitate was collected and resuspended in 3.3 ml of Buffer B. This fraction was then applied to a Sephadex G-25 column (45-ml, 2-cm diameter) equilibrated in and eluted with Buffer B. The pooled Sephadex G-25 void volume protein (Fraction III, 7.5 ml) was applied to a DNA-cellulose column (12 ml, 4-cm diameter) equilibrated in Buffer B. The column was washed with 100 ml of Buffer B and the protein n activity was eluted with 40 ml of Buffer B containing 2 M NaCl. The eluate was precipitated with ammonium sulfate (0.226 g/ml) and dissolved in Buffer D (1 mM dithiothreitol) (Fraction IV, 4 ml). This sample was dialyzed against 1 liter of Buffer D (1 mM dithiothreitol) for 12 hours and the precipitate which formed was collected by centrifugation at 41,000 × *g* for 60 min and suspended by homogenizing it in 4 ml of Buffer B (Fraction V). The precipitate was again collected and the wash was repeated. The final protein pellet was resuspended in 2 ml of Buffer B containing 0.25 M NaCl, clarified, and the supernatant (Fraction VI) withdrawn. This fraction was stored in aliquots in liquid nitrogen where it was stable for at least 6 months. At 0° Fraction VI is stable for at least 2 months. Ammonium sulfate precipitates of each of the fractions were stable on storage at 0° for at least 2 weeks.

Fraction	Total units	Protein	Specific activity	Yield	Purification
	× 10^{-3}	*mg/ml*	*units/mg* × 10^{-3}	%	
I. Extract	190	16.7	0.016		
II. Ammonium sulfate[a]	128	2.9	1.3	87	90
III. DEAE-cellulose	48	6.4	1.0	34	70
IV. DNA-cellulose	35	2.3	3.8	24	250
V. Low ionic strength precipitation	29	0.36	20	20	1,320
VI. Wash	12	0.17	34	8	2,160

[a] Calculation of yield and purification was based on the activity recovered in all of the ammonium sulfate fractions; assays in Fraction I were not reliable.

TABLE VIII

Summary of protein properties

Protein	DNA binding[a]	NEM-sensitive	Gene locus	Molecular weight	Function
*dna*C	+	+	*dna*C	20,000	?[b]
*dna*B	−	−	*dna*B	250,000[c]	?
Protein i	±	−	?	40,000	?
Protein n	+	+	?	80,000	DNA binding
DNA polymerase III holoenzyme[d]	+	+	*dna*E	330,000[e]	DNA synthesis
*dna*G	+	−	*dna*G	65,000[f]	RNA synthesis
DNA-unwinding protein	+	−	?	76,000[g]	DNA binding

[a] Denatured calf thymus DNA-cellulose and 50 mM imidazole-HCl (pH 7.0).

[b] ?, unknown.

[c] Ref. 4.

[d] DNA polymerase III* (*dna*E locus) + copolymerase III* (77,000-dalton subunit, gene locus unknown).

[e] Ref. 12.

[f] Footnote 2.

[g] Ref. 11.

for ϕX174 are dispensable (8). It seems reasonable that *dna*G protein would perform the RNA synthetase function of ϕX174 DNA as it does for G4 DNA and that the four additional proteins may serve by orienting regions of the ϕX174 DNA into a form suitable as an origin ("promoter" for RNA synthesis).

Current studies of a complex of protein n with ϕX174 DNA show a dramatic change in conformation of the DNA. It is reduced 6- to 7-fold in contour length and appears as a string of beads, represented by about 25 molecules of protein n. As for the functions of protein i, *dna*B protein, and *dna*C protein nothing is known as yet. Protein i, like protein n and the DNA unwinding protein, is required in the reaction at a level of approximately 25 molecules/DNA circle; the *dna*B protein may be needed at a lower ratio and is known to have an ATPase activity stimulated by DNA (14). Probably some or all of these proteins interact physically to form the assembly unit required for the initiation events.

Among the eight proteins discussed, four are still unassigned to genetic loci. Mutants defective in proteins i or n, copolymerase III*, or the DNA unwinding protein have yet to be discovered or may be identified with one of the recently reported *dna*H (15), *dna*I (16), or *dna*Z (17) thermosensitive replication mutants. The product of the *dna*A gene of the established loci for DNA replication, is not among those needed for reconstituting the ϕX174 conversion. Nevertheless a dependence on a *dna*A gene product was clearly implied in earlier complementation studies with unfractionated cell extracts (2). Its function may prove to be indirect, such as the neutralization of an inhibitory effect of the defective *dna*A product contributed by the mutant extract.

The requirement for spermidine, as well as for the DNA unwinding protein (8) is puzzling in view of the antagonistic functions these molecules generally perform. Binding of spermidine to DNA, which favors helix formation may be essential for maintaining a certain conformation at a particular stage in the reaction, whereas the unwinding protein may serve by binding the single-stranded part of the molecule for other operations in the reaction. Whether the participation of either the polyamine or the unwinding protein in this enzyme system is an accurate guide to their physiological roles must remain undecided. The fully single-stranded DNA template provided in the *in vitro* system is not found in the cell. Instead decapsidation of the phage particle and penetration of the DNA into the cell is tightly coupled to its replication. Current studies designed to reconstitute the molecular details of these early stages of infection may clarify some of these questions.

Note Added in Proof—Procedures for stabilization and purification of the *dna*C protein now yield a preparation about 20% pure with a specific activity of 80,000 units/mg (see Table IV for comparison).

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