Identification of Bacteriophage T4 Gene 60 Product and a Role for This Protein in DNA Topoisomerase*

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Bacteriophage T4 DNA topoisomerase has been isolated and shown to contain the proteins coded by the DNA-delay genes 39 and 52 (Liu, L. F., Liu, C.-C., and Alberts, B. M. (1979) Nature (Lond.) 281, 456-461 and Stetler, G. L., King, G. J., and Huang, W. M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3737-3741). From complementation measurements in vitro and from earlier genetic evidence, these workers suggested that the product of gene 60 (p60) was also a component of the DNA topoisomerase complex. This paper now establishes the identity of p60 and unequivocally shows that this protein is a component of the enzyme complex.

T4 DNA topoisomerase was purified by a simplified two-column procedure and found to be a stable complex of p39, p52, and a protein with a relative molecular weight of 18,000. The 18,000-dalton chain has been unambiguously shown to be the product of gene 60 through the use of an amber mutant of gene 60 with Sup+ and Sup- hosts and analyses by two-dimensional gel electrophoresis. While p39 and p52 were tightly associated in the wild type enzyme complex, they were readily separated on a hydroxylapatite column from extracts of cells infected by an amber mutant of gene 60. These findings suggest that p60 plays a structural/functional role in the enzyme complex by holding the larger p39 and p52 in juxtaposition.

Liu et al. (2) and Stetler et al. (3) have made the important finding that certain of the DNA-delay genes of bacteriophage T4 code for a new type II DNA topoisomerase, confirming a prediction of McCarthy (4) that these gene products form an enzyme with gyrase-like activity. This DNA topoisomerase is ATP-dependent and relaxes either negative or positive supercoils. T4 DNA topoisomerase activity in extracts was shown to be dependent on the functioning of three T4 DNA-delay genes, 39, 52, and 60 (2). However, while p39 and p52 were shown to be part of the purified enzyme complex (2, 3), the gene 60 product was not clearly identified nor was it unequivocally shown to be part of the complex.

Concurrently with these reports, this laboratory had found that mutants in the T4 DNA-delay genes cause a decrease in the rate of synthesis of deoxyribonucleotides (5). As part of an investigation of this phenomenon and to examine the interaction of DNA topoisomerase with the membrane (6, 7), we embarked on a study of this enzyme.

In this paper, the protein product of gene 60 has been identified by two-dimensional electrophoresis using an amber mutant of the gene and examining cell extracts after infection of Sup+ and Sup- hosts. The phage T4 DNA topoisomerase complex has been isolated by a simplified procedure and shown to contain p60 as an integral part of the enzyme with p39 and p52. In the absence of p60, the other two component proteins do not remain tightly associated.

EXPERIMENTAL PROCEDURES

Biologicals—The Escherichia coli B strain was described earlier (8). E. coli B40, a B strain carrying supD suppressor, was obtained from Larry Snyder, Michigan State University, East Lansing, MI. The bacteriophage T4 mutants used in this study and their sources are listed in Table I. The amHA9 amN82 double mutant was constructed by a standard phage cross in E. coli B40 (9).

Materials—Hydroxyapatite, Bio-Gel HTP, was obtained from Bio-Rad; DEAE-cellulose was DE-52 from Whatman; agarose was SeaKem from Marine Colloids Div., FMC Corp., Rockland, ME; ethidium bromide was from Aldrich; bovine pancreas trypsin, soybean trypsin inhibitor, β-lactoglobulin B, egg-white lysozyme, Brij 58, and PMSF were from Sigma; sperm whale myoglobin was from Schwarz/ Mann; and CHAPS was from Pierce Chemical Co. The other molecular weight marker proteins and all the materials for electrophoresis and autoradiography were obtained as previously described (10).

Bacteriophage T4 Infection and Radioactive Labeling of T4 Early Proteins—The growth media and the methods used for labeled and unlabeled infections by phage T4 have been described (10). Infections by amHA9 amN82 were carried out for 30 min at 30 °C. To label the T4 proteins, 35SO42− was added 2 min after infection to a separate culture, equivalent in volume to about 0.1 of the unlabeled infected culture, at a final activity of 50-100 μCi/ml. Cells infected by amHA9 amN82 will incorporate approximately 50% of the 35SO42− into protein in 30 min under the conditions described (10).

Gel Electrophoresis and Autoradiography—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (11) with the following modifications. The thickness of the slab gels was 1.5 mm and the heights of the separating and stacking gels were 9.6 and 1.0 cm, respectively. The proteins were fixed in the gel in a solution of 10% acetic acid and 25% isopropanol, stained for 1–2 h in 0.08% Coomassie brilliant blue R, and destained in 10% acetic acid. Eastman Kodak XAR-5 film was employed for autoradiography of the dried gels. Samples for separation by one-dimensional electrophoresis were brought to a final concentration of 0.05 M Tris-HCl (pH 6.8), 1% SDS, 1% mercaptoethanol, 4.7% glycerol, and 0.01% bromphenol blue, and the mixtures were heated for approximately 3 min in a boiling water bath. The method of sample preparation for two-dimensional electrophoresis and the conditions for nonequilibrium pH gradient electrophoresis in the first dimension have been previously described (10).

The proteins used as molecular weight markers and their assigned

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1 The abbreviations used are: p39, p52, p60, pipIII, protein products of the corresponding genes; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio]propane sulfonate.
TABLE I

| Bacteriophage T4 strains | Mutant | Gene | Source |
|-------------------------|--------|------|--------|
| amN82                   | 44     | A    |        |
| amHA9                   | ipII   | B    |        |
| amN55                   | 42     | C    |        |
| SP62 amN55              | regA, 42 | C   |        |
| amE594                  | 60     | D    |        |
| amE429                  | 60     | E    |        |
| amE300                  | 60     | E    |        |

Sources are: A. R. S. Edgar, California Institute of Technology Collection; B. L. Black, University of Maryland, Baltimore, MD; C. J. S. Berger, University of Rochester, Rochester, NY; D. H. Bernstein, University of Arizona, Tucson, AZ; E. W. B. Wood, University of Colorado, Boulder, CO.

weights in SDS-polyacrylamide gels are: β-galactosidase, 135,000; phosphorylase a, 92,500; bovine serum albumin, 67,000; pyruvate kinase, 57,000; ovalbumin, 45,000; aldolase, 40,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; DNase I, 31,000; chymotrypsinogen A, 25,700; trypsin, 23,500; soybean trypsin inhibitor, 20,100; β-lactoglobulin B, 18,400; and myoglobin, 17,200.

Analytical Methods—DNA topoisomerase was assayed as described (2), except that bovine serum albumin was substituted for human serum albumin and supercoiled pBR322 DNA was used as the substrate rather than supercoiled PM2 DNA molecules. The protein concentrations were determined by the methods of Kalb and Bernlohr (12) for crude extracts and Sedmak and Grossberg (13) for the purified fractions using bovine serum albumin uncorrected for water as a standard.

Purification of T4 DNA Topoisomerase—Four 1-liter cultures of E. coli B grown to 5×10^9 cells/ml in a rotary shaker bath were infected by T4 phage amHA9 amN82 at multiplicities of 8 at 30 °C, and the infections were allowed to continue for 30 min. The resulting cell pellet was combined with that from 350 ml of cells infected in the same manner but labeled with ^32SO_4^- at 2 min after infection. The combined cells were resuspended in 20 ml of a solution of 40 mM Tris-HCl buffer (pH 7.8 at 25 °C), 2 mM EDTA, 24% (w/w) sucrose (n = 1.37051), and 0.63 mg of egg-white lysozyme/ml, and the suspension was incubated at 0 °C for 1 h. An equal volume of a solution consisting of 1% Brij 58, 40 mM Tris-HCl at pH 7.8, and 20 mM β-mercaptoethanol was added, and the solution was stirred in an ice bath for 1.5-2 h. The lysed extract was centrifuged at 30,000 × g for 1 h at 4 °C, and the supernatant fluid (33.0 ml) was loaded on a DEAE-cellulose column (0.7 × 13 cm) which had been equilibrated with a solution of 40 mM Tris-HCl buffer at pH 7.8, 10 mM β-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol. The unadsorbed fraction was applied directly to a hydroxyapatite column (0.7 × 5.5 cm) equilibrated with a solution of 10% glycerol, 10 mM β-mercaptoethanol and 20 mM potassium phosphate buffer at pH 7.2 (25 °C). After the column was rinsed with equilibration buffer and washed with a solution containing 10% glycerol, 10 mM β-mercaptoethanol, and 0.3 mM potassium phosphate buffer, the enzyme was eluted with a mixture of 10% glycerol, 10 mM β-mercaptoethanol, and 0.5 mM potassium phosphate buffer. The fraction containing the peak enzyme activity and radioactivity was brought to 50% in glycerol and stored at −20 °C. This purification is a modification of the procedure of Liu et al. (2) in which several of their steps are deleted.

RESULTS

Purification of T4 DNA Topoisomerase—All steps were carried out at 4 °C unless otherwise indicated. An outline of the results of the purification is presented in Table II. The data in this table and in Fig. 2B are presented in terms of total protein while the remaining figures include only radiolabeled T4 early proteins. In two chromatographic steps, a yield of 5×10^9 units of T4 DNA topoisomerase activity in 200 μg of protein was obtained from the 30,000 × g supernatant of the crude extract prepared from 4 liters of amHA9 amN82-infected E. coli culture. If the 30,000 × g pellet is further extracted with a solution containing 2 mM NaCl, 50 mM Tris-HCl, pH 7.8, and 0.2% Brij 58 and centrifuged at 100,000 × g for 30 min as described by Stetler et al. (3), an additional 1×10^9 units of purified enzyme can be realized from the dialyzed supernatant by the same purification scheme starting at the DEAE-cellulose column step. In the purification presented in Table II, the specific activity of the T4 DNA topoisomerase product was 2.5×10^9 units/mg of protein. The activities in Fractions 1-III were not measured because of interference by nucleases.

When T4 DNA topoisomerase was purified from amN82- or SP62 amN55-infected cells, an additional protein having a subunit molecular weight of 24,000 by SDS-polyacrylamide electrophoresis accounted for approximately 40% of the radio-labeled protein in the purified fraction. It could be separated from the topoisomerase complex by either a carboxymethyl-

TABLE II

| Purification of phage T4 DNA topoisomerase | Volume | Unit | Protein | Total | Topoisomerase activity |
|-------------------------------------------|--------|------|---------|-------|-----------------------|
| I. Crude extract*                         | 40.3   | 19.5 | 786     |       |                       |
| II. Supernatant                           | 33.0   | 11.7 | 386     |       |                       |
| III. DEAE-cellulose                       | 41.8   | 6.3  | 263     |       |                       |
| IV. Hydroxyapatite*                       | 2.3    | 0.086| 0.20    | 5×10^7| 2.5×10^7              |

*a Prepared from 4 liters of cells infected by T4 amHA9 amN82 (ipIII - dna  ) as described under "Experimental Procedures."
cellulose or a gel filtration column (data not shown). This very basic protein was identified as the protein product of the ipIII gene by two-dimensional gel electrophoresis (10) using the purified protein product of ipIII and amHA9, an amber mutant of ipIII, both from Dr. Lindsay Black, University of Maryland, Baltimore. The protein product of ipIII corresponds closely in size to a protein found in the purified topoisomerase enzyme described in an earlier study (2). This protein appeared to copurify with the enzyme complex through the two chromatographic steps in this procedure, although it was not established whether an interaction between pipIII and the enzyme complex occurs. A double mutant, amHA9 amN82, was constructed to obviate the removal of pipIII, and Fig. 1 compares the purified T4 topoisomerase fractions isolated after infections by ipIII+ dna− and ipIII− dna+ phage.

Fig. 2A presents an analysis of the 35S-labeled proteins found at each successive step in the purification as monitored by electrophoresis on a 12% polyacrylamide gel and subsequent autoradiography. Lane 6 shows 3 major protein bands corresponding to the products of genes 39, 52, and 60 and representing greater than 92% of the total radioactivity in the purified fraction. In Fig. 2B, the proteins in the purified T4 topoisomerase fraction (A, lane 6) have been stained with Coomassie blue. The protein products of genes 39, 52, and 60 are estimated to be 80% of the total stained protein in the gel. Two host proteins having relative molecular weights of 39,000 and 17,000 are present as minor contaminants in this topoisomerase preparation.

The molecular weights of p39, p52, and p60 have been determined by comparison with protein standards of accepted molecular weights on one-dimensional SDS-polyacrylamide gels. The subunit molecular weights of the p39 and p52 chains were determined to be 56,500 and 48,000, respectively, with a 10% polyacrylamide gel (Fig. 3). However, it should be noted that membrane proteins may give molecular weight estimates from SDS-gel electrophoresis which are not a direct measure of polypeptide molecular weight since hydrophobic proteins may bind greater than normal amounts of SDS (14, 15). Using a 12% polyacrylamide gel, the protein product of gene 60 was shown to have a molecular weight of 18,000 (Fig. 4). The 16,000-dalton chain associated with the purified T4 topoisomerase described by Liu et al. (2) most probably was p60 based on the enzymatic activity of their preparation and our identification of p60 by two-dimensional gel electrophoresis (see below).

Studies with the purified T4 topoisomerase have suggested that it is a stable complex. The three subunits of the complex remained associated when sedimented on a linear sucrose gradient (5-20%, w/w). In this experiment, the native topoisomerase complex migrated between catalase (M, = 243,000 (16)) and aldolase (M, = 160,000) (data not shown). The three proteins also remained associated when extracted from the membrane with a solution of 2 M NaCl and 0.2% Brij 58. The complex did not dissociate in the pH range of 5.1 to 8.0 or in the presence of 5 mM CHAPS, a highly disaggregating detergent used for protein solubilization (17, 18). These preliminary studies suggest that the three proteins are held together both by hydrophobic and ionic forces.

Identification of Components of T4 DNA Topoisomerase Using Two-dimensional Gel Electrophoresis—Two-dimen-
sional separation by nonequilibrium pH gradient electrophoresis followed by SDS-polyacrylamide electrophoresis has been used to identify 17 bacteriophage T4 prereplicative proteins (10). The protein products of genes 39 and 52 were identified in E. coli B cultures infected by amN116 and amH17, respectively, and their locations are indicated on the gel pattern (amN55 infection) shown in Fig. 5. Two-dimensional gel electrophoresis of the crude extract from E. coli B (Sup−) cells infected by the gene 60 amber mutant, E594,

**Fig. 4.** Determination of the molecular weight of p60 by electrophoretic mobility of an SDS-polyacrylamide gel. A 12% polyacrylamide gel was employed.

**Fig. 5.** Identification of p60. This experiment employed two-dimensional gel electrophoresis of 35S-labeled proteins synthesized after infections by phage T4 gene 42 and gene 60 mutants. In the infections by the amber mutant of gene 60, Sup− and Sup+ hosts were used. Infections were for 8 min at 30 °C. The autoradiogram at the top of the figure is from a two-dimensional gel of T4 early proteins synthesized after infection by amN55 (gene 42). The proteins corresponding to p39 and p52 are labeled at the left edge of the spots. The spot marked by an arrow corresponds to p60. A and B include only the area outlined in black in the top gel pattern. A shows the early proteins synthesized after infection of E. coli B cells by amE594 (gene 60), and the arrow marks the position of the missing spot corresponding to p60. B shows the early proteins synthesized after infection of E. coli B40 (supD) cells by amE594, and the arrow indicates the point of reappearance of a protein with slightly altered mobility corresponding to the suppressed p60. The autoradiograms in A and B are overexposed in comparison to the amN55 gel pattern to clearly demonstrate the disappearance and reappearance of the p60 spot.

**Fig. 6.** Purified T4 DNA topoisomerase. This autoradiogram shows the two-dimensional electrophoretic separation of the 35S-labeled proteins eluted from a hydroxylapatite column with 0.5 M potassium phosphate buffer in the purification of T4 DNA topoisomerase from amHA9 amN82-infected E. coli B cells.
demonstrated that an 18,000-dalton protein chain was absent by comparison to the gel pattern obtained on infection with amN55 (Fig. 5A). When amE594 was used to infect the E. coli B amber suppressor strain, B40, a protein chain reappeared which had the same charge but migrated slightly slower than the wild type gene 60 product (Fig. 5B). Because of the nature of the suppressor mechanism, this slower mobility is undoubtedly the result of the amino acid substitution by the suppressor and not an alteration in chain size. An amino acid substitution can alter the hydrophobicity of a protein and thereby change the migration of the suppressed gene product (19, 20). The identification of the gene 60 protein product was confirmed by two-dimensional gel analysis of crude extracts from two other gene 60 amber mutants, amE300 and amE429. Both lacked the protein spot identified as p60, but contained wild type amounts of p39 and p52 (data not shown).

Fig. 6 shows a two-dimensional separation of purified T4 DNA topoisomerase. When the autoradiograms from the purified enzyme and crude extracts are aligned, the three 35S-labeled protein chains correspond exactly to the spots identified as p39, p52, and p60. The two-dimensional gel patterns established that gene 60 codes for a protein chain of 18,000 daltons and that this protein is present in the purified T4 DNA topoisomerase with p39 and p52.

\[ \text{p60 Plays a Structural/Functional Role in the T4 DNA Topoisomerase Complex} \]

As mentioned previously, the T4 DNA topoisomerase complex is rather stable and does not readily dissociate into its subunits. However, when extracts of E. coli B cells infected by an amber mutant of gene 60 were carried through the purification scheme outlined in Table II, p39 and p52 no longer eluted together from the hydroxylapatite column with 0.5 M potassium phosphate buffer. The autoradiogram in Fig. 7 shows the 35S-labeled proteins eluted from the hydroxylapatite column at varying potassium phosphate concentrations after infection by the gene 60 amber mutant, amE429 or amE594. In each case, p52 was eluted with 0.3 M potassium phosphate buffer, while p39 bound more tightly to the column and was eluted only after increasing the potassium phosphate concentration to 0.5 M. The identifications of p39 and p52 were confirmed by analysis of the fractions eluted by 0.3 and 0.5 M potassium phosphate using two-dimensional gel electrophoresia. The 35S-labeled protein having a molecular weight of 44,500 and eluting with p39 in the 0.5 M potassium phosphate buffer has been shown to be a proteolytic fragment of p39 by the method of Cleveland et al. (21) (data not shown).

These experiments give clear evidence that p60 has a structural role in the T4 DNA topoisomerase complex, i.e. that this subunit is directly involved in the association of p39 and p52.

**DISCUSSION**

This work has characterized the protein product of gene 60 and established that p60 is part of T4 DNA topoisomerase and has a structural/functional role in the enzyme complex. The primary findings are as follows: 1) p60 has been identified by two-dimensional gel electrophoresia and shown to be an 18,000-dalton protein chain; 2) T4 DNA topoisomerase has been purified by a simplified procedure. The purified enzyme is a stable complex and was shown to contain p39, p52, and p60 by both one- and two-dimensional gel electrophoresia; 3) in the absence of p60, the other two proteins, p39 and p52, did not remain associated but were readily separated on a hydroxylapatite column.

The finding that p60 is required for the tight association of p39 and p52 suggests that this small protein chain serves as a structural link, perhaps providing flexibility to the enzyme while holding the larger chains in juxtaposition. Preliminary studies show that the enzyme complex sediments in sucrose gradients between catalase and aldolase. The coaxial ratio of the molecule will need to be considered (22), but on the basis of the precedence of an $\alpha_2\beta_2$ structure for the host enzyme (23–25), the observations are consistent with p60 (a) or its dimer acting as a linker between p39 (a) and p52 (b) in the T4 enzyme with a structure such as $\alpha_2\gamma=\beta_2$.

It is appropriate to review the differences between the phage-coded enzyme and its comparable type II DNA topoisomerase in the host. Liu et al. (2) and Stettler et al. (3) have shown that the phage enzyme relaxes positive or negative supercoils, whereas the host enzyme has the unique ability to introduce negative supercoils (26). The host enzyme dissociates readily into its $\alpha_2$ and $\beta_2$ subunits (27), whereas the isolation procedures of Stettler et al. and of Liu et al. and our own attempts at dissociation demonstrate that the phage T4 enzyme is a tight complex. Whether the third protein chain in the T4 enzyme is related to these differences remains to be determined.

The studies presented have led to procedures to separate
the three protein chains of T4 DNA topoisomerase in pure form so that each can be studied in terms of its interaction with synthetic phospholipid vesicles, binding to DNA, and activity as an ATPase.

T4 DNA topoisomerase may have other functions in addition to its proposed role in replication or in initiation of replication (2, 3, 28-31). Not only has this enzyme been implicated in deoxyribonucleotide synthesis in vivo (5), but it has also been found in quantity in preparations of T4-induced deoxyribonucleotide synthetase complex (32). Recent experiments have shown that T4 topoisomerase may have a role in the synthesis of the β subunit of T4 ribonucleoside diphosphate reductase (33), perhaps by mechanisms similar to those proposed for DNA topoisomerase I of E. coli in the transcription of several genes (34).

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