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T4 DNA-delay proteins, required for specific DNA replication, form a complex that has ATP-dependent DNA topoisomerase activity

(cellophane discs/*in vitro* complementation assays/superhelical DNA/DNA untwisting)

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ABSTRACT **Under some conditions, T4 DNA replication requires the products of the DNA-delay genes, genes 39, 52, 58, and 60. By using an *in vitro* complementation assay that stimulates DNA replication in T4 39(−)-infected cell extracts, T4 gene 39 protein has been purified. The purified fraction also contains complementing activities for T4 genes 52 and 60. On sodium dodecyl sulfate/polyacrylamide gel analysis the purifed preparation exhibits three protein components: a 51,000-dalton protein corresponding to the product of gene 52, a 64,000-dalton protein corresponding to the product of gene 39, and a 110,000-dalton protein. This purified fraction shows a DNA topoisomerase activity that untwists superhelical DNA in an ATP- and Mg^{2+}-dependent reaction. The analogs adenylyl imidodiphosphate and adenyl [β,γ-methylene]diphosphonate cannot be used to replace ATP. The topoisomerase activity is not sensitive to the antibiotics oxolinic acid and novobiocin, known antagonists of *Escherichia coli* DNA gyrase. The possible relationship among the three polypeptides and their biological activities is discussed.**

DNA replication for even the simple bacteriophages is a complex process involving the joint participation of many proteins, RNA, and possibly membrane (1). For the large bacteriophage T4, at least 20 essential phage genes are needed for DNA replication and metabolism (2). Among them there are phage genes defined by a set of mutants called "DNA-delay" mutants in genes 39, 52, 58, and 60 (3). These mutants are so named because they are characterized by a delay in the onset of phage DNA replication. In the absence of any one of these gene products, phage DNA replication is not completely abolished. This phenotype is to be distinguished from that of mutants in the genes whose products are absolutely required for chain elongation and fork movement—namely, the proteins of genes 32, 41, 43, 44, 62, and 45 (4). However, in the absence of more than one DNA-delay protein, the delay time in the onset of DNA replication is lengthened (5). In the absence of gene 39, 52, and 58 proteins, DNA replication is so delayed that it is effectively aborted (unpublished observation), even though the other replication proteins required for chain elongation and fork movement are present in normal amounts. This evidence suggests that normal T4 DNA replication does require the presence of DNA-delay proteins.

The products of DNA-delay genes 39 and 52 have been identified on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels as specific protein bands of 64,000 and 51,000 daltons, respectively (6). These two proteins have been shown to be associated with the bacterial membrane (7), and there is independent evidence suggesting that T4 DNA replication occurs on the membrane (8). Furthermore, these two proteins have been shown to bind DNA tightly (9). More recently, it has been shown that T4 gene 39 protein may function in positively regulating the specific replication of T4 DNA *in vivo* (10). The apparent phenotype of the DNA-delay mutants and the physical properties of the DNA-delay proteins suggest that these gene products may be involved in the process of initiating T4 DNA replication at the origin.

Recently, an *in vitro* T4 DNA replication system using semipermeable membrane discs as a means to prepare highly concentrated extracts has been developed (11). This system mimics many of the characteristics of the *in vivo* replication process, including the requirements for DNA-delay proteins. By using this *in vitro* DNA synthesizing system, specific DNA-delay proteins can be studied by a complementation assay that measures the stimulation of DNA synthesis in extracts lacking the DNA-delay proteins. In this report, we describe the purification of the products of DNA-delay genes 39 and 52. The purified fraction, which contains three major protein subunits, has an ATP-dependent DNA topoisomerase activity that untwists superhelical DNA.

MATERIALS AND METHODS

Bacterial and Phage Strains. *Escherichia coli* B was used as the nonpermissive host for T4 amber mutants. B-40 (suI) or CR63 (suI) served as the permissive host. JT1200 (polA) was used to prepare the recipient extracts for the complementation assays for the DNA-delay proteins. All T4 phages are from the collection originally maintained at the California Institute of Technology.

Chemicals. All nucleoside triphosphates and UDP-glucose were obtained from P-L Biochemicals. [^{3}H]dTTP was obtained from New England Nuclear. The 5-hydroxymethyl derivative of dCTP ($HOCH_2dCTP$) was prepared enzymatically according to established procedures (12). Lysozyme was from Worthington. Polyethyleneimine was purchased from Miles. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad. Nalidixic acid and novobiocin were purchased from Sigma. Oxolinic acid was a gift of Warner-Lambert Research Institute. Derivatives of pBR322 plasmid DNA were purified from a cleared lysate and subjected to CsCl/ethidium bromide equilibrium centrifugation as described (13).

Enzyme Assays. The detailed procedure of the complementation assay for T4 DNA-delay proteins will be published elsewhere. Briefly, the assay measures the stimulation of DNA synthesis *in vitro* using, as recipients, extracts prepared from cultures infected with amber mutants defective in the DNA-

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Abbreviations: $HOCH_2dCTP$, 5-hydroxymethyl derivative of dCTP; p[NH]ppA, adenylyl imidodiphosphate (adenosine 5′-[β,γ-imido]-triphosphate); p[CH_2]ppA, adenylyl [β,γ-methylene]diphosphonate; $NaDodSO_4$, sodium dodecyl sulfate.

delay genes. The recipient extracts were very gently lysed with lysozome and detergent BRIJ-58 and then were spread on semipermeable cellophane discs (14). The complementation reaction mixture (0.06 ml) contained 2×10^8 equivalents of infected cells, 1 mM ATP, UTP, CTP, and GTP each at 0.055 mM, 0.17 mM each of dATP, $HOCH_2$dCTP, dGTP, dTTP (^{3}H-labeled, 2.5 μCi/nmol; 1 Ci = 3.7×10^{10} becquerels) each at 0.17 mM, 0.3 mM UDP-glucose, 20 mM morpholinopropane sulfonic acid (pH 7.5), 5 mM $MgCl_2$, 0.1 M KCl, 17 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, and 50 μg of nalidixic acid per ml. Incubation was at 20°C, and the amount of DNA synthesized was measured as acid-insoluble material. One unit of DNA-delay protein stimulates the incorporation of 10 pmol of dTMP under these conditions in 30 min at 20°C.

The DNA topoisomerase assay measures the relaxation of supercoiled pBR322 DNA or its derivatives as monitored by agarose gel electrophoresis (15). The reaction mixture (0.025 ml) contained 35 mM Tris·HCl (pH 7.5), 7 mM $MgCl_2$, 35 mM potassium phosphate, 25 mM NaCl, 1 mM dithiothreitol, 0.25 mM ATP, 20% (wt/vol) glycerol, and 1 μg of DNA. Incubation was at 20°C. At the end of the incubation period, the reaction was stopped by the addition of EDTA to a final concentration of 45 mM. The mixture was subjected to analysis by electrophoresis in 0.7% agarose gel, and DNA was visualized under UV light after staining with ethidium bromide.

Other Methods. The analysis of protein content by electrophoresis using a slab of 10% polyacrylamide gel containing $NaDodSO_4$ has been described (6). The procedure for labeling T4 early proteins has also been reported (6). Protein concentrations were determined by the method of Lowry *et al.* (16).

Table 1. Purification of T4 gene 39 protein

Step	Protein, mg	Activity, units	Specific activity, units/mg
1. BRIJ lysate	7600	—	—
2. Membrane fraction	1260	1×10^6	7.9×10^2
3. Salt extraction	250	1×10^6	4.0×10^3
4. Polymin P	47	3×10^5	6.4×10^3
5. Hydroxylapatite	0.082	9×10^4	1.1×10^6

T4 am4314 (gene 43)-infected cells (40 liters) were harvested and lysed by lysozyme and BRIJ as described (7) (fraction 1). All operations were performed at 0–4°C and all buffers contained 5 mM 2-mercaptoethanol, 2 mM EDTA, and 5% glycerol except where indicated. The lysed extract was centrifuged for 60 min at 27,000 × *g* to remove the supernatant from the membrane pellet (fraction 2). Fraction 2 was extracted by gentle stirring for 4 hr with 30 ml of 2 M NaCl/50 mM Tris·HCl, pH 7.5/0.2% BRIJ-58. The suspension was then centrifuged at 100,000 × *g* for 30 min in a Beckman SW-27 rotor. After a dialysis against 20 mM Tris·HCl, pH 7.5/0.1 M NaCl, the preparation was adjusted to 50 ml with the dialysis buffer (fraction 3). Fraction 3 was precipitated with 3.5 ml of 10% (vol/vol) polyethyleneimine and the precipitate was extracted with 10 ml of 20 mM Tris·HCl, pH 7.6/1 M NaCl. The supernatant was adjusted to 0.1 M in Tris·HCl at pH 7.5, and solid $(NH_4)_2SO_4$ was added to 30% saturation. After the removal of insoluble material, the supernatant was further adjusted to 50% saturation with solid $(NH_4)_2SO_4$. The suspension was gently stirred for 1½ hr and then centrifuged at 100,000 × *g* for 1 hr in a Beckman SW-27 rotor. The pellet was dissolved in 1.5 ml of 20 mM potassium phosphate, pH 7.0/0.1 M NaCl (fraction 4, 3.1 ml). After dialysis against 20 mM potassium phosphate/0.1 M NaCl, fraction 4 was applied to a column (2 × 28 cm) of hydroxylapatite. The column was eluted with a 300-ml linear gradient of 0.1–0.8 M potassium phosphate containing 0.1 M NaCl. A portion of the active material (fraction 5) was dialyzed against 20 mM potassium phosphate, pH 7.0/0.1 M NaCl/25% glycerol and applied to a small hydroxylapatite column (bed volume, 0.5 ml). The sample was eluted with 0.5 M potassium phosphate, pH 7/0.1 M NaCl/25% glycerol. The fractions containing active T4 gene 39 protein were pooled, adjusted to 50% glycerol, and stored at −20°C.

RESULTS

Purification of T4 Gene 39 Protein. T4 gene 39 protein was purified from am4314 (gene 43)-infected cells which carry the T4 gene 39 on a plasmid (17). As an assay for the purification, we used the ability to stimulate DNA synthesis *in vitro* in a 39(−) mutant (amN116)-infected recipient on cellophane discs. The procedure for the purification is described in Table 1. T4 gene 39 protein was purified approximately 1400-fold; the analysis of the purified material by $NaDodSO_4$/polyacrylamide gel electrophoresis is shown in Fig. 1. After staining with Coomassie blue, fraction 5 revealed three polypeptide chains with apparent molecular weights of 110,000, 64,000, and 51,000. These proteins were further studied by comparing their electrophoretic mobilities with those of T4 early proteins labeled with amino acid precursor and analyzed on the same slab gel. The band at molecular weight 64,000 corresponded to the product of gene 39 because it was missing from the T4 extract infected with an amber mutant defective in gene 39. Similarly,

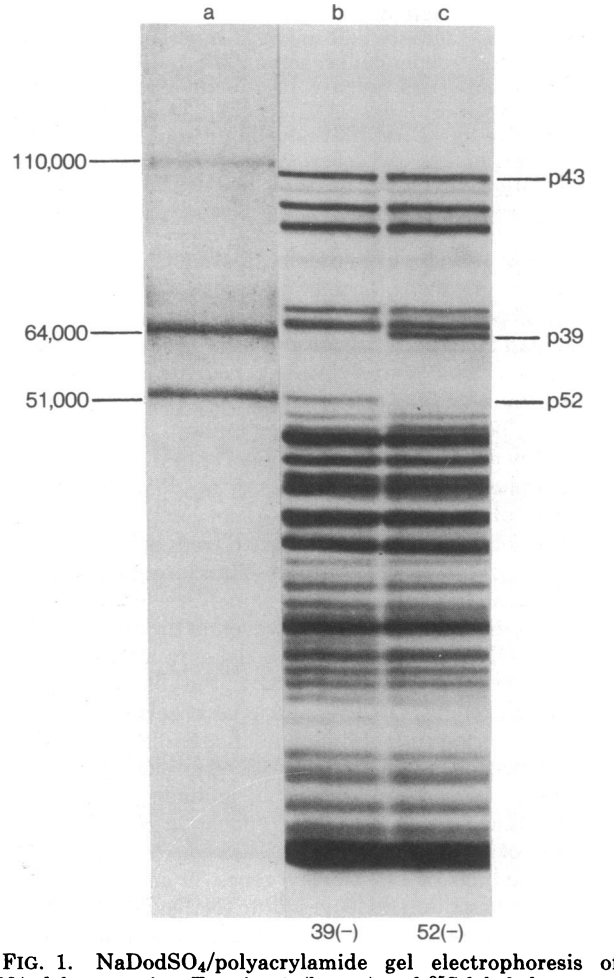

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of DNA-delay proteins. Fraction 5 (lane a) and ^{35}S-labeled extracts prepared from T4 39(−) (am N116) mutant-infected cells (lane b) and T4 52(−) (am H17) mutant-infected cells (lane c) were analyzed on a slab of 10% polyacrylamide gel containing $NaDodSO_4$. At the end of electrophoresis, the gel was first stained with Coomassie blue and then subjected to autoradiography. Lanes b and c are the autoradiographic patterns of T4 early proteins. p43, p39, and p52 designate the positions of the products of T4 genes 43 (T4 DNA polymerase), 39, and 52.

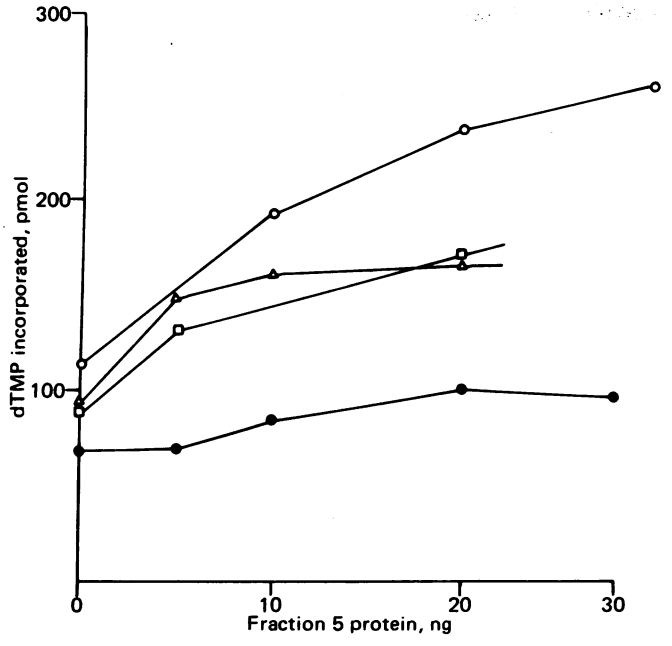

FIG. 2. Complementation in T4-infected cell extracts. Varying amounts of fraction 5 protein were added to extracts prepared from T4 mutants defective in DNA-delay genes, and DNA synthesis on cellophane discs was measured. T4 mutants used were: □, 39(−) (am N116); ○, 52(−) (am H17); ●, 58(−) (am HL627); and △, 60(−) (am E429).

the protein band of molecular weight 51,000 corresponded to the product of gene 52. These assignments are in complete agreement with an earlier report (6). It should be noted that the amount of the molecular weight 110,000 peptide present in fraction 5 is much less than the amount of the other two peptides (it represents about 10% of the total protein). Thus far, we have not been able to achieve further separation of these three proteins from fraction 5 by conventional methods without significant loss of gene 39 protein complementation activity.

Other DNA-Delay Protein Complementing Activities. Fraction 5 was also tested for its ability to stimulate DNA synthesis in extracts prepared from other T4 DNA-delay genes (genes 52, 58, and 60). Fig. 2 shows that, in addition to gene 39 protein complementing activity, fraction 5 contained activities to stimulate DNA synthesis in extracts prepared from 52(−)- and 60(−)-infected cells but only weak activity in 58(−) extract. Because the presence of gene 39 and 52 proteins in fraction 5 had been shown by $NaDodSO_4$/polyacrylamide gel, the observed complementing activities of the gene 39 and 52 protein in fraction 5 can be attributed to the 64,000- and 51,000-dalton bands, respectively. The protein bands corresponding to genes 60 and 58 have not been identified on $NaDodSO_4$ gels. Because T4 extracts prepared from 60(−) mutant-infected cells do contain normal levels of gene 39 and 52 proteins when analyzed by $NaDodSO_4$ gel electrophoresis (data not shown), the ability of fraction 5 to stimulate DNA synthesis in a 60(−) recipient extract is not likely to be due to the 39 or 52 proteins. Therefore, it is possible that the observed gene 60 protein complementing activity may be related to the 110,000-dalton protein band, but we cannot rule out the possibility that it is due to the presence of another minor protein not revealed by the Coomassie blue stain.

Purified DNA-Delay Protein Complex Has a DNA Topoisomerase Activity That Untwists DNA. When highly supercoiled DNA was incubated with varying amounts of protein from fraction 5, the DNA became relaxed to form closed circular molecules with fewer superhelical turns (Fig. 3*A*). The kinetics of the DNA relaxation is shown in Fig. 3*B*. The enzyme complex appears to act processively because the distributions of the relaxed DNA species remained the same when either enzyme or substrate (supercoiled DNA) was in excess.

The Topoisomerase Activity Is Resistant to Oxolinic Acid and Novobiocin. The complementing activities of the DNA-delay proteins are resistant to nalidixic acid (50 μg/ml), included in the incubation mixture for routine assays. T4 DNA-delay protein complementation activities also are resistant to oxolinic acid and novobiocin (unpublished observation). When

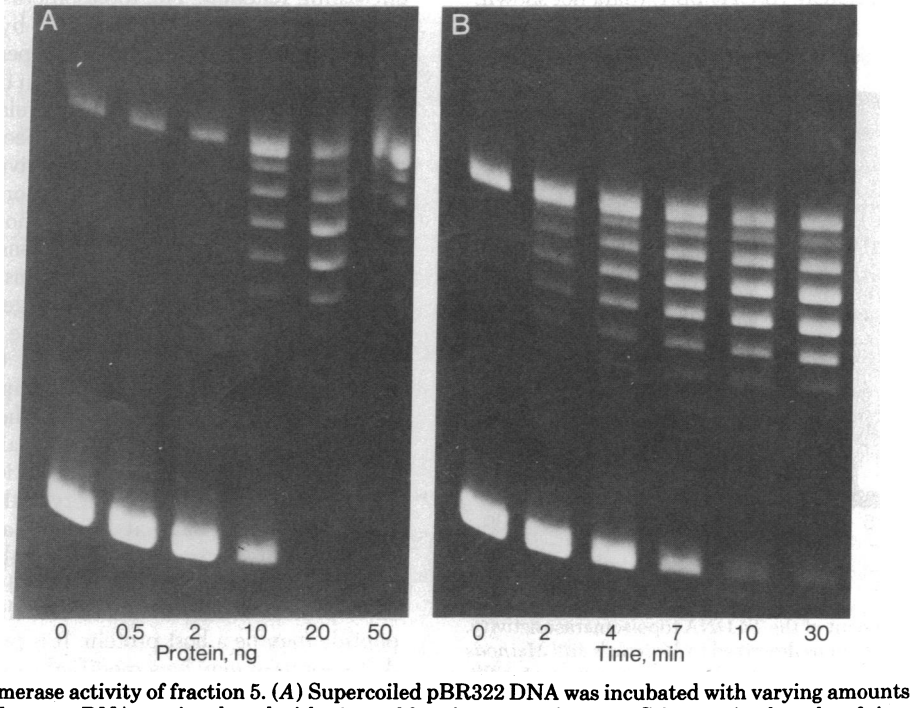

FIG. 3. DNA topoisomerase activity of fraction 5. (*A*) Supercoiled pBR322 DNA was incubated with varying amounts of fraction 5 protein for 30 min at 20°C. (*B*) The same DNA was incubated with 10 ng of fraction 5 protein at 20°C for varying lengths of time.

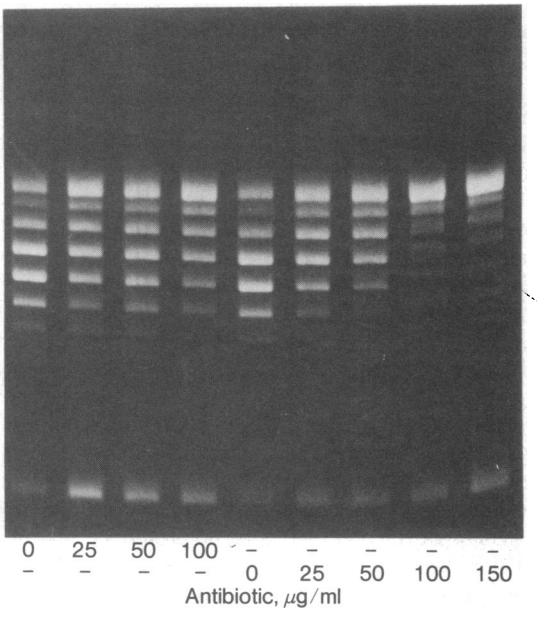

FIG. 4. Sensitivity of the DNA-topoisomerase activity to novobiocin (upper line) and oxolinic acid (lower line).

these drugs were added to the incubation mixtures, topoisomerase activity also was resistant even at concentrations above 100 μg/ml (Fig. 4). These are levels comparable to the amount necessary to inactivate *E. coli* DNA gyrase (18, 19). It should be noted that the distribution of the relaxed circles formed in the presence of oxolinic acid, but not with novobiocin, was qualitatively different from that in its absence.

T4 DNA Topoisomerase Activity Requires ATP. Unlike the nicking-closing topoisomerase activity of DNA gyrase, rat liver untwisting enzyme, and ω protein (20–24), the relaxation of supercoiled DNA by the T4 DNA-delay proteins has an absolute requirement for ATP (Fig. 5). Furthermore, the ATP analogs adenylyl imidodiphosphate (p[NH]ppA) (Fig. 5) and adenylyl [β,γ-methylene]diphosphonate (p[CH_2]ppA) (data not shown)

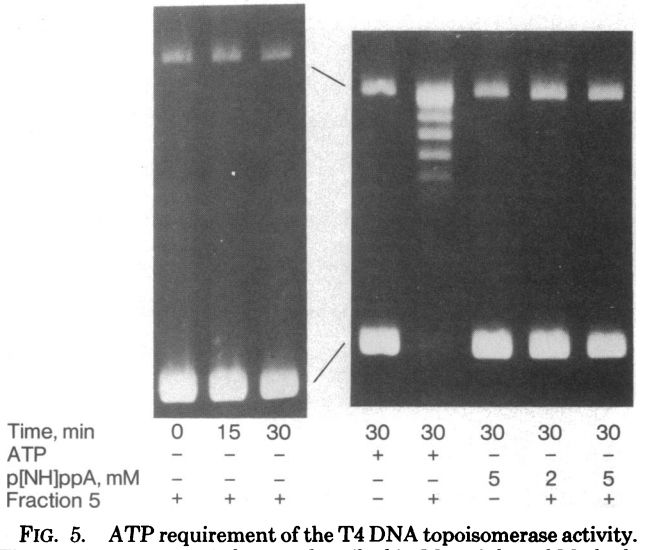

FIG. 5. ATP requirement of the T4 DNA topoisomerase activity. The reactions were carried out as described in *Materials and Methods* except where omissions are indicated. The concentration of ATP, when added, was 0.25 mM. The incubation mixture contained 10 ng of fraction 5 protein when added.

even at high concentrations (5 mM) could not be used to replace ATP. Therefore, it appears that ATP utilization (possibly hydrolysis) is coupled to the DNA relaxation activity, and it is a special feature of the T4 DNA-delay proteins not shared by any other known DNA untwisting enzymes. The topoisomerase activity of DNA-delay protein also required Mg^{2+} under our assay conditions (data not shown).

DISCUSSION

Using a protein complementation assay that measures the stimulation of DNA synthesis in a T4 39(−) mutant-infected extract, we have purified T4 gene 39 protein approximately 1400-fold. The most highly purified fraction was found to consist of three polypeptides. Two of these polypeptides could be identified as the products of genes 39 and 52 based on their complementation activities as well as their band assignments on $NaDodSO_4$/polyacrylamide gel. The third component, revealed by the $NaDodSO_4$/polyacrylamide gel analysis is a protein of molecular weight 110,000. The copurification of these three polypeptides suggests that they may form a complex. This is supported by the following observations. (*i*) Using conventional methods of separation, we have been unable to resolve the 39 and 52 proteins without significant loss of their biological activities. (*ii*) When the active fractions were analyzed on glycerol gradients, the three polypeptides sedimented together as 8–10S material as analyzed by $NaDodSO_4$/polyacrylamide gels (data not shown). (*iii*) The purified preparation (fraction 5) containing the three polypeptides exhibits a unique topoisomerase activity that untwists superhelical DNA. A similar activity has also been found in crude extracts by Liu *et al.* (L. F. Liu, C.-C. Liu, and B. M. Alberts, personal communication). This topoisomerase activity is absent in 39(−), 52(−), or 60(−) mutant-infected cell extracts.

The purified T4 DNA-delay proteins (fraction 5) catalyze the untwisting of superhelical DNA in an ATP-dependent reaction. The ATP dependence is not shared by the other known DNA "swivelases" (20). The ATP requirement cannot be satisfied by analogs of ATP such as p[NH]ppA and p[CH_2]ppA, which implies that ATP hydrolysis may be coupled with the untwisting reaction. The topoisomerase activity we have reported here could also be explained by the concerted actions of an endonuclease and an ATP-dependent ligase. Our data show that this is not the case. First, in the absence of ATP, superhelical DNA is not converted to relaxed (or nicked) circles by the purified preparation (Fig. 5). Second, fraction 5 does not contain detectable T4 DNA ligase activity. When open circular DNA (either singly nicked with pancreatic DNase or treated with *Eco*RI) was used as a substrate, no detectable ligation was found under our incubation conditions (data not shown).

Our purified preparation contains three different DNA complementing activities—those of genes 39, 52, and 60. The data presented here strongly indicate the involvement of T4 genes 39 and 52 proteins in the T4 topoisomerase activity. However, the role of the gene 60 complementing activity and the 110,000-dalton polypeptide and their relationship with the observed topoisomerase activity remain unclear. It seems unlikely that the 110,000-dalton polypeptide is the product of gene 60, because we have never observed the induction of an early T4 phage protein band at 110,000 daltons when radioactive amino acid precursors were added at the time of infection. (T4 DNA polymerase, molecular weight 105,000, is the largest T4 early protein induced.) Therefore, the 110,000-dalton polypeptide may be a host protein. It is possible that the 110,000-dalton protein may be a modified form of subunit A of the *E. coli* DNA gyrase (pnaI protein) or *E. coli* ω protein, because they all have the same molecular weight (18, 21). *E. coli* gyrase

and ω protein both catalyze an ATP-independent topoisomerase reaction that untwists DNA. In the T4 case, the topoisomerase activity is ATP-dependent and oxolinic acid resistant. These special features may be acquired by complexing the *E. coli* protein with T4 proteins (39 and 52 proteins) or by other modifications.

It is interesting to note that T4 DNA replication, normally only weakly sensitive to both oxolinic acid and novobiocin, becomes more sensitive to these drugs in mutants defective in DNA-delay genes. This observation can be demonstrated both *in vivo* (25) and *in vitro* (unpublished data). This makes the possible involvement of an *E. coli* DNA gyrase subunit in the T4 activity even more attractive. Our present working model is that, after T4 infection, T4 52 and 39 proteins form a complex (possibly together with an *E. coli* host protein such as a gyrase subunit) to catalyze the ATP-dependent topoisomerase activity. Formation of such a complex may be catalyzed or modified by gene 60. In this way we may account for the presence in the purified fraction of three complementing activities (those of genes 39, 52, and 60) and three polypeptides (the 39 protein, the 52 protein, and the 110,000-dalton protein).

The involvement of a DNA topoisomerase in the replication of circular DNA molecules such as plasmid DNA and φX174 replicative factor is well documented (reviewed in ref. 26). It has also become clear that the chain elongation process at the replication fork is not sufficient to drive the unwinding process; a topoisomerase activity has been proposed to be present ahead of the fork to facilitate the unwinding (27). In the case of T4, DNA replication has been implicated to occur on the bacterial membrane. If this is true, then the anchoring of the duplex DNA to the membrane at more than one point might create topological constraints on the DNA helix, making a topoisomerase required for DNA replication and at the initiation (or onset) of DNA replication. This idea is supported by the observation that T4 DNA-delay proteins are required for replication and our results reported here showing that the DNA-delay protein complex is a topoisomerase. The understanding, at the molecular level, of how a general topoisomerase capable of functioning with any superhelical DNA is used to regulate specific T4 replication remains to be elucidated.

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