T4 DNA topoisomerase: a new ATP-dependent enzyme essential for initiation of T4 bacteriophage DNA replication

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A novel ATP-dependent DNA topoisomerase which makes reversible double-strand breaks in the DNA double helix has been purified to near homogeneity from T4 bacteriophage-infected Escherichia coli *cells. Genetic data suggest that this activity is essential for initiating T4 DNA replication forks* in vivo.

THE process of DNA replication fork movement can be successfully duplicated *in vitro* and has been shown to require several different types of purified protein[1,2]. In contrast, the process of replication intiation, whereby new replication forks are established at specific chromosome origins, has not yet been reconstituted with purified components. As a result, our knowledge of the enzymology of fork initiation is rather limited (for review see ref. 3).

Genetic studies of T4 bacteriophage development have indicated that the products of T4 genes *39*, *52* and *60* probably interact with each other to form a complex, and that this complex is essential for normal T4 DNA replication[4]. When any one of these three T4 proteins is defective, replication still begins at about the same time as in the wild type, but the rate of the initial phase of DNA synthesis is reduced[5]. This slow rate of DNA synthesis results from a low incidence of replicative forks, rather than from any reduction of the fork movement rate[6]. The products of genes *39*, *52* and *60* are thus believed to be involved in the initiation of T4 DNA replication[6].

Here, we report the discovery, properties and purification of a new ATP-dependent DNA topoisomerase. DNA topoisomerase is the name suggested for a class of enzymes that catalyse the concerted breaking and rejoining of DNA backbone bonds, as these activities are invariably detected by their ability to promote the interconversion between different topological isomers of DNA (for review, see ref. 7). The T4-induced DNA topoisomerase characterised here seems to be composed of proteins coded for by T4 genes *39*, *52* and *60*. The properties of this enzyme suggest ways in which it can act to initiate replication forks at T4 chromosome origins. A similar complex of T4 proteins has been independently isolated by Stetler, King and Huang[8], using an *in vitro* complementation assay to monitor gene *39* protein activity.

A new ATP-dependent DNA topoisomerase activity induced by T4 bacteriophage infection

Infection of *Escherichia coli* with T4 bacteriophage results in a marked increase in the ability of a cell lysate to relax covalently closed superhelical DNAs, a reaction which requires at least one transient cleavage of a DNA backbone bond[9]. This conversion of DNA circles from a negatively supercoiled form to a less supercoiled form may be sensitively detected by agarose gel electrophoresis, as shown in Fig. 1 for circular duplex PM2 DNA which has been incubated with various dilutions of an extract. Supercoil-relaxing activity is detected in this crude lysate only after extensive dilution away from the endonucleases which are also present; these nucleases convert the initial DNA substrate (form I DNA) to nicked DNA circles (form II DNA) and unit-length linear DNA molecules (form III DNA). For this reason, a group of DNA bands characteristic of partially relaxed, covalently closed DNA circles (which have an electrophoretic mobility between that of form I and form II DNAs) could be detected only after a 540-fold extract dilution. Surprisingly, no DNA supercoil relaxation was detected when the rATP in the reaction mixture was omitted. This activity was not blocked by antibody to *E. coli* ω protein or affected by the *E. coli* DNA gyrase inhibitor novobiocin (data not shown). The possibility of DNA relaxation by an excess of DNA ligase in the presence of an endonuclease was ruled out, as the same level of supercoil-relaxing activity was found in extracts of cells infected with a T4 DNA ligase (gene *30*) amber mutant (see Table 1). We therefore concluded that the activity detected in Fig. 1 originates from a previously unrecognised DNA topoisomerase. By analogy with other DNA topoisomerases, we assume that this enzyme catalyses both the breakage and the rejoining of DNA backbone bonds; moreover, it must do so in a concerted way, such that the putative intermediate in which a DNA backbone bond is broken exists only transiently. This requirement is necessitated by our failure to detect a population of nicked DNA circles as intermediates in reactions with the purified enzyme (see below).

T4 mutants of genes 39, 52 and 60 eliminate topoisomerase activity

A large number of T4 mutants were screened for the ability to affect or abolish the ATP-dependent DNA topoisomerase activity seen in Fig. 1. For this purpose, sonically disrupted cell extracts were prepared from a variety of T4 mutant-infected cells. The mutants screened for activity are listed in Table 1; of these only *am*N116 (gene 39^-), *am*H17 (gene 52^-) and *am*HL626 (gene 60^-) showed no detectable DNA topoisomerase activity in extracts. As expected, the activity was missing when these amber mutant phages were used to infect a non-suppressing (su^-) *E. coli* host (*E. coli* B); activity was present when the same mutants were used to infect an amber-suppressor strain (*E. coli* CR63). Furthermore, when any two of these three amber mutants were used to co-infect the non-suppressing *E. coli* host (*E. coli* B), the topoisomerase activity was largely recovered (see Table 1). Thus, the genetic complementation observed between mutants in these three genes *in vivo* is matched by activity complementation for the

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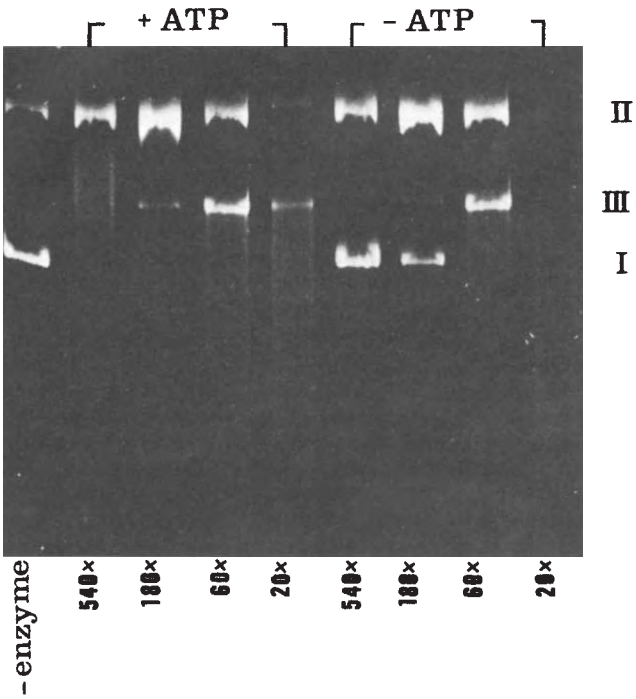


Fig. 1 An ATP-dependent DNA topoisomerase activity can be detected in extensively diluted crude extracts of T4 bacteriophage-infected *E. coli*. The Roman numerals on the right-hand margin indicate the positions after agarose gel electrophoresis of form I (covalently closed, negatively supercoiled duplex DNA circle), form II (nicked DNA circle) and form III (unit-length linear) PM2 DNA molecules. A completely relaxed closed circular DNA would co-migrate with form II DNA on this gel. The bands migrating between form II and form III DNAs in the second lane from the left (adjacent to the no enzyme control) are the covalently closed DNA circles which have a reduced superhelical density due to topoisomerase action. Note that cleavages by endogeneous nucleases produce form II and form III DNAs at high extract concentrations preventing the detection of the topoisomerase activity. Reaction mixtures (20 μl each) contained 50 mM Tris-HCl, *p*H 7.8, 40 mM KCl, 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_3EDTA, 30 μg ml^{-1} human serum albumin, 25 μg ml^{-1} covalently closed circular PM2 DNA and 0.5mM ATP (where indicated). To this mixture, 1 μl of extract diluted to various extents was added to start the reaction; the numbers below each gel lane represent the final dilution of the original extract in the 20-μl reaction. After 30 min at 30 °C, each reaction was stopped by adding 5 μl of 50% glycerol, 5% SDS and 1 mg ml^{-1} bromphenol blue and loaded on a 0.7% agarose gel. The gel was electrophoresed at room temperature in a buffer consisting of 90 mM Tris-borate (*p*H 8.3) and 5 mM $MgCl_2$ for about 15 h at 2.5 V cm^{-1}. To visualise the DNA, the gel was stained with 1 μg ml^{-1} ethidium bromide, visualised on a short-wave UV transilluminator and photographed with Polaroid film type 55. The source of infected cells for these experiments was *E. coli* strain D110 (thy$^-$, polA$^-$, endI$^-$), which had been infected for 30 min at 37 °C with a MOI of 10 of T4 bacteriophage [*reg*A-*am*N55(*42*$^-$)-*am*H39(*30*$^-$)] in M9 minimal medium supplemented with 0.3% casein hydrolysate, 1 μg ml^{-1} thiamine and 10 μg ml^{-1} thymidine. The infected cells (5×10^8 cells ml^{-1}) were chilled, washed and concentrated 800-fold by centrifugation. After Brij lysis[29], the lysate was centrifuged at high speed (60 min at 35,000*g*), and the supernatant was used as the extract.

topoisomerase in extracts. Note, however, that the identification of the gene 60 product as being involved here is made less clear by complications arising from *E. coli* B–*E. coli* K host-range differences (see refs 4, 5).

Because infection of *E. coli* B with T4 amber mutants in genes *39*, *52* or *60* causes a major delay in DNA synthesis, they have been designated as 'DNA-delay' (DD) mutants[10]. DNA-delay amber mutants infecting su$^-$ *E. coli* eventually produce a substantial burst of progeny phage, although reduced relative to wild-type infection. This suggests that growth of the DNA-delay mutants on a su$^-$ *E. coli* depends on a host function which is only partially effective[4]. Using drug inhibition studies, McCarthy recently demonstrated that one compensating host factor is *E. coli* DNA gyrase, as, unlike wild-type T4, the growth of T4 gene *39*, *52* and *60* mutants is very sensitive to the DNA gyrase inhibitors coumermycin and novobiocin[11,12]. Using autoradiography to measure the replication fork rates during gene *52* mutant infections, McCarthy *et al.*[6] have further suggested that the DNA-delay mutants are defective only in the initiation of T4 DNA replication, as the fork rates observed are normal.

Table 1 T4 mutants in genes *39*, *52* and *60* eliminate the T4 DNA topoisomerase activity

T4 mutant	Relative T4 DNA topoisomerase activity Su$^-$ *E. coli*	Su$^+$ *E. coli*
Uninfected	<5	<5
*am*H39 (*30*$^-$)	(100)	(100)
*an*N116 (*39*$^-$)	<5	25
*am*H17 (*52*$^-$)	<5	25
*am*HL626 (*60*$^-$)	<5	100
*am*N116 (*39*$^-$) + *am*H17 (*52*$^-$) co-infection	20	NT
*am*H17 (*52*$^-$) + *am*HL626 (*60*$^-$) co-infection	15	NT
*am*HL626 (*60*$^-$) + *am*N116 (*39*$^-$) co-infection	30	NT

Both *E. coli* CR63 (su$^+$) and *E. coli* B (su$^-$) were grown at 37 °C in 20 ml of H broth to a cell density of 5×10^8 cells ml^{-1}; each culture was then infected at a MOI of 10, for each of the indicated T4 bacteriophages. At 12 min after infection, the cells were chilled, pelleted and resuspended in 320 μl of 50 mM Tris-HCl, *p*H 7.8, 50 mM KCl, 10 mM β-mercaptoethanol and 1 mM Na_3EDTA. Each batch of cells was broken by sonication and centrifuged for 15 min at 8,000*g* in an Eppendorf model 3200 centrifuge. Serial dilutions of each supernatant were then assayed as described in Fig. 1 legend, except that 0.2 mg ml^{-1} naladixic acid was present in each assay. The activity in each extract was estimated by comparing the relaxation activity with that in serial dilutions of the T4-*am*H39 (gene *30*$^-$, DNA ligase) infected *E. coli* B extract, whose activity was taken as 100. Other mutants tested, with activities comparable to wild type were *am*N134-*am*BL292-*am*E219 (*33*$^-$, *55*$^-$, *61*$^-$), SP62-*am*N55-*am*H39 (*reg*A$^-$, *42*$^-$, *30*$^-$), *am*HL628 (*59*$^-$), *am*N011 (*47*$^-$), *am*N130 (*46*$^-$)-*am*E727 (*49*$^-$), and the large non-essential deletions: Δ(far P13), Δ(63–32) and $\Delta(39\text{–}56)_{12}$-alc$^-$-ΔSaΔ9(ac–denB)-nd28(denA). NT, not tested.

Purified T4 DNA topoisomerase has multiple components

Starting from 100 g of T4-infected *E. coli* cells, 0.8 mg of highly purified T4 DNA topoisomerase was obtained following the multi-step procedure given in Fig. 2 legend. The total purification, estimated from specific activity measurement, was 350-fold, with a yield of 5%. With a unit of activity defined as the amount of enzyme necessary to induce half-relaxation of 0.3 μg of pBR322 DNA in 30 min at 30 °C in our standard reaction mixture (see Fig. 1 legend), the final DNA–cellulose-purified T4 DNA topoisomerase had a specific activity of 10^6 units per mg. As revealed by the Coomassie-stained SDS-polyacrylamide gel shown in Fig. 2, this fraction contains two major protein bands (molecular weights 63,000 and 52,000), which comprise about 70% of the total stained protein in the gel. Based on previous molecular weight estimates[13], these two bands are almost certainly the products of T4 genes *39* (MW assigned at 64,000) and *52* (MW assigned at 51,000), respectively. Two other protein bands with MWs of 23,000 and 16,000 are also present on our gels. Of these two species, only the 16,000-MW protein actually co-purifies with the topoisomerase activity (data not shown). In view of the data presented in Table 1, and the genetic evidence that the gene products of *39*, *52* and *60* interact with each other *in vivo*, it is tempting to identify the 16,000-MW protein as the T4 gene 60 product.

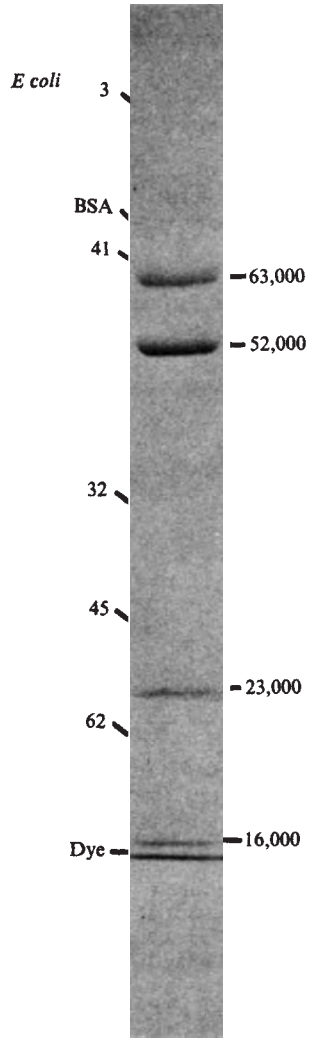


Fig. 2 SDS-polyacrylamide gel analysis of highly purified T4 DNA topoisomerase. A total of 9 μg of purified T4 DNA topoisomerase was denatured in SDS and electrophoresed on a 10% polyacrylamide slab gel[30]; the resulting protein bands were then stained with Coomassie blue as described elsewhere[31]. The approximate polypeptide MWs observed are indicated on the right hand margin, and the migration positions for other purified T4 replication proteins[29,32] used as markers (listed by T4 gene numbers), and for the *E. coli* ω protein[7], are indicated on the left hand margin. To purify the enzyme, frozen T4 (*reg*A-*am*N55-*am*H39) infected *E. coli* D110 cells (100 g) were thawed and resuspended in 150 ml of buffer containing 40 mM Tris HCl, *p*H 7.8, 2 mM Na_3EDTA, 25% sucrose and 95 mg of egg-white lysozyme (Worthington). After 60 min at 0 °C, the cells were lysed by adding 150 ml of 1% (w/v) Brij 58, 40 mM Tris-HCl and 20 mM β-mercaptoethanol and stirring at 0 °C for another 60 min. After centrifugation at 30,000*g* for 1 h, the 330 ml of supernatant was loaded on to a DEAE-cellulose (Worthington DE-52) column (2.5 cm × 24 cm) equilibrated with buffer A (40 mM Tris HCl, *p*H 7.8, 10 mM β-mercaptoethanol, 1 mM Na_3EDTA and 10% (w/v) glycerol). The flow-through fractions were pooled and precipitated by gradual addition of 10% (w/v) polymin P solution (*p*H 7.9) to a final concentration of 0.3%. The precipitate was collected by centrifugation (23,000*g* for 15 min), and the topoisomerase activity was then solubilised by extraction with 100 ml of 0.2 M NaCl (in buffer A). The topoisomerase was precipitated by ammonium sulphate (50 g per 100 ml), collected by centrifugation and redissolved in 30 ml of buffer A. After dialysis overnight against 2 l of buffer A, the topoisomerase was loaded on to a second DE-52 column (2.5 cm × 8 cm) equilibrated with buffer A. The flow through from this column was loaded directly on to a hydroxyapatite column (2.5 cm × 8 cm) equilibrated with solution B (10% glycerol, 10 mM β-mercaptoethanol) containing 20 mM potassium phosphate (*p*H 7.2). After washing the column with solution B containing 0.3 M potassium phosphate, the topoisomerase activity was eluted with solution B containing 0.5 M potassium phosphate. Pooled fractions with activity were dialysed into buffer A containing 50 mM KCl and loaded at 4 ml h^{-1} on to a DNA–cellulose column (1.2 cm × 3 cm containing 1 mg per packed ml of single-stranded calf thymus DNA). This column was eluted successively with buffer A containing 50 mM, 100 mM, 200 mM and 300 mM KCl. The topoisomerase appeared in the 200 mM KCl eluate. The peak activity was pooled and dialysed against a buffer consisting of 30 mM potassium phosphate (*p*H 7.2), 50% (w/v) glycerol, 10 mM mercaptoethanol and 0.5 mM Na_3EDTA, for storage at −20 °C, at which it seems to be quite stable.

The purified T4 DNA topoisomerase can be shown to act catalytically: one enzyme molecule (assumed to have a MW of 131,000) is capable of relaxing to completion at least seven molecules of pBR322 DNA in a 30-min incubation. The topoisomerase activity is completely resistant to novobiocin (up to 60 μg ml^{-1}), but is weakly sensitive to high concentrations of nalidixic acid. This inhibitory effect of nalidixic acid was further studied by using oxolinic acid, a more potent analogue of nalidixic acid; the T4 DNA topoisomerase activity was halved by oxolinic acid at about 250 μg ml^{-1}. As this level of oxolinic acid is about an order of magnitude higher than the concentrations needed for the inhibition of *E. coli* DNA gyrase[14,15], it is likely that the observed inhibition is nonspecific. A similar 'nonspecific' inhibition has been previously observed for another DNA topoisomerase, *E. coli* ω protein, at comparably high oxolinic acid concentrations (500 μg ml^{-1}, ref. 15). When tested with a variety of different circular DNA molecules, the topoisomerase activity shows no obvious DNA preference. The purified enzyme exhibits no DNA gyrase activity with either fully relaxed pBR322 DNA or PM2 DNA in our assay conditions (that is, it is unable to introduce supercoils into these DNA molecules).

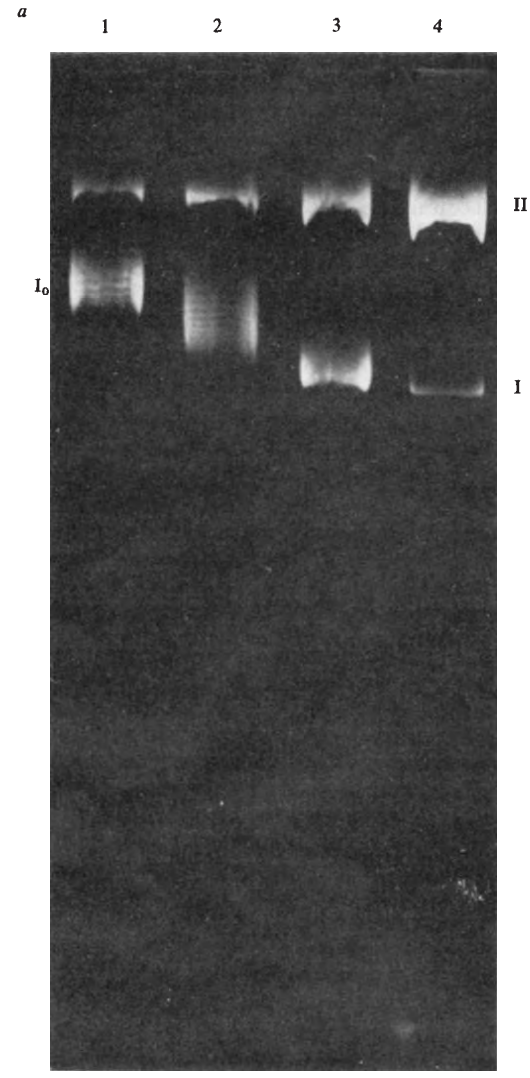
Both in the crude extract and in the first DEAE-cellulose breakthrough, the topoisomerase activity is associated with a 30S complex, as judged by sucrose gradient sedimentation (data not shown). This complex can be destroyed, without affecting the ATP-dependent relaxation activity, either by pancreatic RNase A treatment or by salt-extracting the enzyme from a precipitate formed with the synthetic polycation polymin P, as described in Fig. 2 legend. The significance of this apparent RNA association in crude fractions is not known. However, our purified enzyme contains less than 1% nucleic acid, and it sediments more slowly than 5S.

The topoisomerase can catalyse the ATP-dependent relaxation of positive DNA twists

Unlike the *E. coli* ω protein (which can only remove negative turns), T4 DNA topoisomerase catalyses the relaxation of both negatively and positively superhelical DNAs. To assay for the relaxation of positively supercoiled DNA, a fully relaxed PM2 DNA substrate was prepared, and ethidium bromide was added to make this relaxed DNA act as if positively supercoiled. The relaxation of the positive DNA twists present in these conditions can be detected by the increased mobility of the covalently closed PM2 DNA, seen when this DNA is subjected to electrophoresis in the absence of ethidium bromide (see Fig. 3*a*). With our highly purified T4 DNA topoisomerase, we have also demonstrated that: (1) the relaxation of both negative and positive twists is ATP dependent; (2) both reactions are gradual, indicating that the enzyme does not relax the DNA by a one-hit mechanism and that it dissociates from each DNA molecule before relaxing it completely, (3) the enzyme acts catalytically in both cases, in that one T4 DNA topoisomerase molecule can relax many PM2 DNA molecules in a 30-min reaction (data not shown).

ATP hydrolysis is required for catalytic relaxation

The relaxation of superhelical DNAs of both senses is a thermodynamically favourable process. Correspondingly, none of the relaxation reactions catalysed by the other known DNA topoisomerases requires an exogenous source of energy[7]. In contrast, we have shown (Fig. 1) that the T4-induced DNA topoisomerase requires ATP as a cofactor. The requirement for ATP cannot be replaced by other ribo- or deoxyribonucleoside



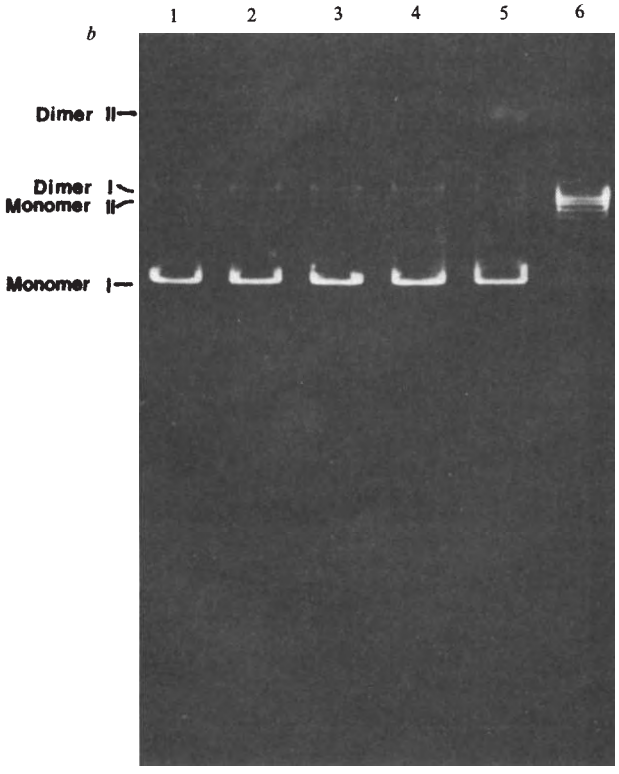


Fig. 3 *a*, The ATP-dependent relaxation of positively superhelical PM2 DNA catalysed by the T4 DNA topoisomerase. Reactions were carried out as described for Fig. 1, except that fully relaxed, covalently closed PM2 DNA (form I_0) was the substrate and ethidium bromide was present at 2 μg ml^{-1} to induce positive superhelical turns[33]. As the source of the topoisomerase, the breakthrough of the initial DE-52 chromatography step (described in Fig. 2 legend) was used. This partially purified topoisomerase was diluted into the reaction mixture as follows: lane 1, no enzyme; lane 2, 2,000-fold; lane 3, 500-fold; lane 4, 100-fold. Electrophoresis was carried out as described for Fig. 1, but at 4 °C. The removal of ethidium bromide during electrophoresis causes the unreacted substrate DNA to return to a relaxed conformation, but a small amount of negative superhelicity is induced by the low temperature used for electrophoresis. As a result, at 4 °C, the substrate DNA (denoted as form I_0) migrates faster than nicked circular DNA (form II), but more slowly than the highly negatively supercoiled form I DNA. In contrast, the DNA relaxed in the presence of ethidium bromide gains a great deal of negative superhelicity on removal of this dye; thus, the final product of the topoisomerase reaction is the rapidly migrating form I DNA. Note that, with increasing enzyme, increasing numbers of negative supercoils are induced by the topoisomerase in all the covalently closed DNA circles which survive the incubation; this change in superhelicity is only obtained when ATP is present (data not shown). In addition, the topoisomerase still contains an endonuclease contaminant at this stage, which causes the amount of form II DNA to increase as more enzyme is added. *b*, When inducing DNA relaxation, the topoisomerase acts catalytically and requires ATP hydrolysis. Reaction mixtures (20 μl each) contained 50 mM Tris-HCl, *p*H 7.8, 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_3EDTA, 30 μg ml^{-1} human serum albumin, 20 μg ml^{-1} form I pBR322 DNA (negatively supercoiled) and 0.2 μg ml^{-1} of the most highly purified fraction of T4 DNA topoisomerase. Incubations were carried out for 30 min at 30 °C, and the products were analysed as described for Fig. 1. Lane 1 is the no enzyme control; lanes 2–6 represent reactions carried out with 70 μM rATP present, plus different amounts of the non-hydrolysable ATP analogue, ATP-γS, as follows: lane 2, 500 μM; lane 3, 200 μM; lane 4, 50 μM; lane 5, 10 μM; lane 6, no ATP-γS. As nearly complete inhibition of the topoisomerase activity occurs with 10 μM ATP-γS in the presence of 70 μM ATP (lane 5), it is clear that the K_i for ATP-γS is much lower than the K_m for ATP.

triphosphates. This surprising energy requirement has prompted us to investigate the ATP dependence further.

The fact that actual ATP hydrolysis (rather than merely the presence of ATP) is required comes from two lines of evidence. First, the compound rATP-γS [riboadenosine 5′-*O*-(3-thiotriphosphate)] inhibits the topoisomerase reaction strongly when added to our normal reaction mix, as shown in Fig. 3*b*. rATP-γS is an analogue of rATP which is poorly hydrolysed by enzymes which cleave the β–γ phosphodiester linkage of ATP. It acts as an inhibitor of many ATPases, including the activities of the T4 gene *41* and *44/62* proteins (unpublished observation of this laboratory). Second, as shown in Table 2, a DNA-dependent ATPase activity can be detected in the most purified fraction of T4 DNA topoisomerase, for which the hydrolysis products are ADP and inorganic phosphate. With certain assumptions (see Table 2 legend), we can calculate that the number of ATP molecules hydrolysed is roughly equal to the number of superhelical DNA turns relaxed by the topoisomerase.

It is important to note that although rATP-γS is a potent inhibitor of the T4 DNA topoisomerase-catalysed relaxation reaction, it can, nonetheless, stimulate the relaxation reaction in the absence of ATP when the enzyme is present at substrate levels (C.-C.L., unpublished results). This situation is analogous to the effect of a non-hydrolysable analogue of ATP, App(NH)p, on *E. coli* DNA gyrase[16]. For gyrase, the binding of ATP seems to induce a conformational change in the protein which leads to a single cycle of DNA supercoiling; the hydrolysis of ATP is then required to return the enzyme to its original conformation[16]. The situation for the T4 DNA topoisomerase is probably similar, except that one cycle of DNA relaxation,

Table 2 DNA-dependent ATPase activity of the T4 DNA topoisomerase

	ATP hydrolysed (nmol ml^{-1})		
	5 min	10 min	15 min
−DNA	<0.2	< 0.2	0.2
+35 μg ml^{-1} ss fd DNA	0.9	1.9	2.5
+20 μg ml^{-1} pBR322 DNA	2.0	4.0	5.0
+50 μg ml^{-1} T4 ds DNA	3.7	8.3	10.5

The reaction mixtures (20 μl each) were as described in Fig. 1 legend, except that 100 μM [γ-^{32}P]ATP was present. The most purified T4 DNA topoisomerase (0.5 μg) was added to each reaction mixture. After incubation at 30 °C for the times indicated, aliquots (50 μl) of reaction mixtures were withdrawn and added to 0.5 ml of 0.1 M HCl. Hydrolysis was measured as inorganic ^{32}P (as phosphate) separated from unhydrolysed [γ-^{32}P]ATP by charcoal adsorption[27]. The different DNAs present in each reaction mixture are indicated. That the products of hydrolysis are ADP and inorganic phosphate was determined in a separate experiment in which ^{3}H-ATP was used as substrate and the products were analysed by chromatography on PEI-cellulose[28]. From the ATP hydrolysis observed during a 30-min incubation at 30 °C using 0.5 μg of enzyme and 100 μM ATP, we calculate that about 1 pmol of ATP would be hydrolysed by 2.5 ng of enzyme (assuming that the dose response is linear for ATPase activity). In the same conditions, 2.5 ng of enzyme will relax about half of the superhelical turns in 0.1 pmol (as DNA molecules) of pBR322 DNA. As there are about 30 superhelical turns in each pBR322 DNA molecule in our reaction conditions, we estimate that one or two superhelical DNA turns are relaxed for every ATP molecule hydrolysed.

rather than DNA supercoiling, is observed on ATP binding. However, we cannot rule out the possibility that the stimulation is due to a very slow hydrolysis of ATP-γS by the topoisomerase.

Similarities between the T4 DNA topoisomerase and *E. coli* DNA gyrase

At least two kinds of DNA topoisomerase are found in bacteria, as exemplified by the *E. coli* ω protein (*Eco* DNA topoisomerase I) and the *E. coli* DNA gyrase (*Eco* DNA topoisomerase II). *E. coli* ω protein catalyses the energetically favourable partial relaxation of negatively superhelical DNA with no added energy cofactor[17]. In contrast, DNA gyrase[36] catalyses the reverse reaction, introducing negative superhelicity into DNA in a reaction requiring ATP hydrolysis[16,18].

The T4 topoisomerase and the *E. coli* gyrase are multiple-subunit proteins which have both nicking–closing and DNA-dependent ATPase activities. In both cases, these two activities are apparently tightly linked, inasmuch as each cycle of nicking and closing needs to be accompanied by hydrolysis of one (or two) ATP molecules[16,18]. Finally, for both enzymes addition of SDS and protease seems to trap nicked DNA–enzyme intermediates, in which both DNA strands have been broken to produce a unit-length linear DNA molecule from a double-stranded DNA circle (refs 14, 15 and L.F.L., unpublished results). Nevertheless, there is at least one important difference: only the *E. coli* gyrase is able to harness its ATP hydrolysis energy to do useful work (induce DNA supercoiling); so far in our experiments the T4 DNA topoisomerase seems to be wasting this energy, as the reaction catalysed is already thermodynamically favourable without ATP hydrolysis.

How might T4 DNA topoisomerase function in replication fork initiation?

The replication machinery of T4 bacteriophage has largely been reconstructed *in vitro*. Using seven highly purified T4 proteins essential for T4 DNa replication *in vivo*, DNA synthesis has been demonstrated to occur efficiently and with high fidelity on double-stranded DNA templates[19]. However, this synthesis begins by a strand displacement reaction which is initiated by covalent addition on to the 3′-end of a pre-existing DNA nick[19]. The expected *de novo* fork initiation, by formation of an *in vivo*-like 'replication bubble' on a double-stranded T4 DNA molecule, has not been achieved in this (or any other) purified *in vitro* system. The previously described genetic evidence implying that the T4 gene *52*, *39* and *60* products are specifically involved in T4 replication fork initiation, coupled with the present identification of these proteins as a topoisomerase, suggests that this enzyme may be the missing factor.

The tight coupling between the ATP hydrolysis and relaxation activities of T4 DNA topoisomerase is unique among all known DNA topoisomerases. Although it is possible that the ATP hydrolysis functions directly in the nicking–closing reaction (for example, by being used to provide energy for ligation of the putative broken DNA chain intermediates), it seems much more likely that the ATP hydrolysis energy is normally coupled to another, as yet unidentified, energy-utilising reaction.

We have no direct evidence as to how ATP energy is used by the topoisomerase at the replication origin. However, we will present a speculative model which is suggested by the above-mentioned similarities between the T4 DNA topoisomerase and DNA gyrase. In this view, the T4 DNA topoisomerase serves as a DNA gyrase-like enzyme which acts specifically at the T4 replication origin. Such an enzyme might function to initiate new T4 replication forks in a manner analogous to that schematically outlined in Fig. 4. Here, the T4 DNA topoisomerase would recognise two specific DNA sequences spanning the origin[20] of T4 DNA replication. By anchoring these two DNA sequences on the same enzyme molecule and thereby restricting the rotation of the DNA strands relative to the enzyme, the topoisomerase could create a closed topological domain. This would allow its hypothetical ATP-driven gyration to induce a local negative supercoiling which destabilises the double helix at the replication origin. As indicated, the melting of this origin would presumably require the gene *32* helix-destabilising protein. Participation of the T4 gene *41* and/or gene *44/62* replication proteins, destabilising the helix by ATP-driven walking

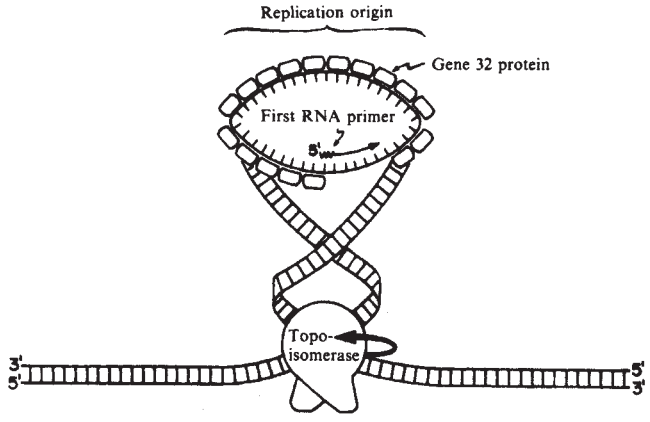


Fig. 4 Two possible models for T4 DNA topoisomerase action at the T4 replication origin. These two models assume (without proof) that the rate-limiting step in generating new replication forks is the opening of the DNA helix at the origin (see text). *a*, Topoisomerase gyration. In this hypothetical scheme, the topoisomerase acts as a site-specific DNA gyrase and uses its ATP hydrolysis and nicking–closing activities to seperate the strands of the DNA helix at the T4 replication origin. The replication origin must form a closed loop, as an isolated domain is required in the otherwise linear T4 DNA molecule to allow supercoiling. This loop could be formed either by the binding of the topoisomerase alone (as indicated here) or by a separate set of proteins or membrane sites. The T4 gene 39 and 52 proteins have been reported to interact with the cell membrane, as well as with DNA[34], and so some membrane attachment might also be used to constrain the DNA origin in this way. (It is interesting that limited topological domains have been detected in the condensed T4 chromosome[35].) In any case, the topoisomerase is suggested to recognise a set of specific DNA sequences which designate a T4 chromosome origin, and to show a DNA gyrase-like activity there (induction of negative twists which destabilise the helix) which is not detected with the non-origin DNA sequences present in our standard topoisomerase assay.

along DNA single-strands[21,22], is also possible. Note that, in this view, the ATP-dependent relaxation of non-origin-containing DNA molecules observed *in vitro* reflects an uncoupled version of a site-specific DNA gyration reaction catalysed by the topoisomerase *in vivo*.

The site-specific gyration model in Fig. 4 postulates that the initial opening of the DNA helix is followed by an RNA-primed DNA chain start, which actually begins DNA synthesis. This first RNA primer could either be synthesised by the same enzymes that prime the Okazaki pieces made on the lagging strand of the T4 replication fork, or be made by a separate mechanism[3,23]. It has been shown recently that the T4 gene *61* protein, in conjunction with the T4 gene *41* protein, can synthesise pentaribonucleotides, and that this synthesis has an absolute requirement for a single-stranded DNA template (ref. 19 and C.-C.L., unpublished results). When additional replication proteins are present, these short ribonucleotides will prime lagging strand DNA synthesis in the T4 *in vitro* system[19]. These two T4 proteins may therefore also have a crucial role in replication fork initiation.

Despite our limited knowledge concerning the mechanism of initiation of DNA replication forks, some similarities can be seen between different systems. As is the case for many other genomes (bacteriophage λ, *E. coli* and eukaryotic chromosomes), replication forks in T4 seem to be able to start out bi-directionally from an initial replication bubble[24]. We therefore expect that a detailed study of the T4 DNA topoisomerase and its interaction with the other T4 replication proteins can provide a model system in which some general properties of fork initiation mechanisms can be elucidated.

Genetic data suggest that genes *O* and *P* of bacteriophage λ may act in a manner analogous to T4 DNA topoisomerase. As for T4 genes *39*, *52* and *60*, the λ *O* and *P* products interact with each other, and when their levels are decreased, the initiation of λ DNA replication is altered[25]. Experiments comparing various lambdoid phages have shown the gene *O* product is highly phage specific, presumably because it recognises origin DNA sequences[26]. Similarly, unlike most T4 genes, gene *39* function cannot be complemented by bacteriophage T2 (W. M. Huang, personal communication); this suggests that gene *39* may have a role in T4 replication which is analogous to the role of gene *O* in λ replication.

Further study of the T4 DNA topoisomerase should also provide valuable knowledge concerning the functions and mechanisms of the important class of enzymes known as DNA topoisomerases. In this respect, we have observed that, at much higher enzyme concentrations (about equal weights of DNA and T4 DNA topoisomerase), a novel ATP-independent DNA topoisomerisation reaction can be detected, in which topologically knotted, double-stranded superhelical DNA molecules are created. Most strikingly, these knots can subsequently be removed by catalytic amounts of the topoisomerase, in an efficient ATP-dependent reaction. As will be described in detail elsewhere, such data reveal that the T4 DNA topoisomerase is working by making transient double-stranded DNA breaks which are rapidly and efficiently resealed in its 'closing' reaction.

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Erratum

In the letter 'Basaltic pillars in collapsed lava-pool on the deep ocean floor' by J. Francheteau *et al.*, *Nature* **281,** 209–211, the third author's name was mis-spelt; it should read C. Rangin. Figures 1 and 2 were incorrectly reproduced. They are shown correctly below.

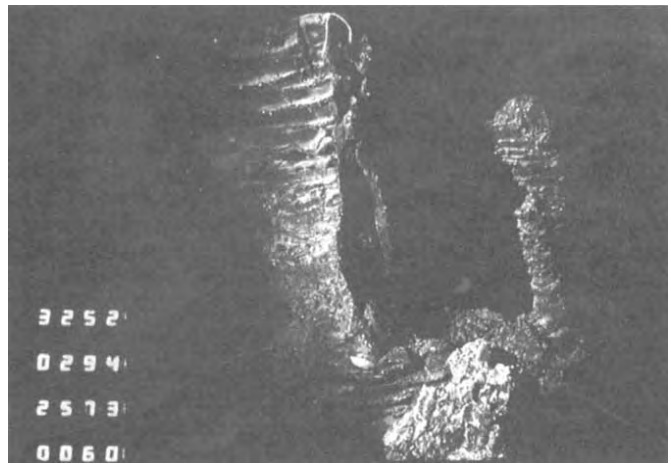

Fig. 1 General landscape: pillars with collapsed lava pond.

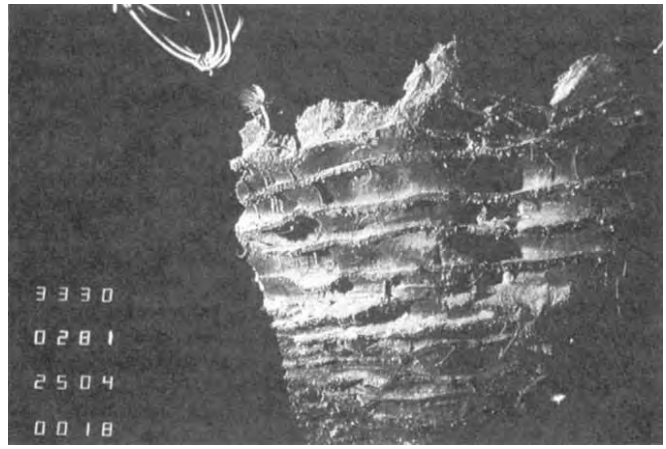

Fig. 2 Detail of a pillar showing the centimetric pseudo layering with thin and darker salient glass layers projecting from the basaltic surface of the pillar.