Site-specific Recognition of Bacteriophage T4 DNA by T4 Type II DNA Topoisomerase and Escherichia coli DNA Gyrase*

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The site specificity of bacteriophage T4-induced type II DNA topoisomerase action on double-stranded DNA has been explored by studying the sites where DNA cleavages are induced by the enzyme. Oxolinic acid addition increases the frequency at which øX174 duplex DNA is cut by the enzyme by about 100-fold, to the point where nearly every topoisomerase molecule causes a double-stranded DNA cleavage event. The effect of oxolinic acid on the enzyme is very similar to its effect on another type II DNA topoisomerase, the Escherichia coli DNA gyrase. A filter-binding method was developed that allows efficient purification of topoisomerase-cleaved DNA fragments by selecting for the covalent attachment of this DNA to the enzyme. Using this method, T4 topoisomerase recognition of mutant cytosine-containing T4 DNA was found to be relatively nonspecific, whereas quite specific recognition sites were observed on native T4 DNA, which contains glucosylated hydroxymethylcytosine residues. The increased specificity of native T4 DNA recognition seems to be due entirely to the glucose modification. In contrast, E. coli DNA gyrase shows a high level of specificity for both the mutant cytosine-containing DNA and native T4 DNA, recognizing about five strong cleavage sites on both substrates. An unexpected feature of DNA recognition by the T4 topoisomerase is that the addition of the cofactor ATP strongly stimulates the topoisomerase-cleaved DNA fragmenting of native T4 DNA, but has only a slight effect on cleavage of cytosine-containing T4 DNA.

Bacteriophage T4 provides one of the best model systems for studies of the mechanism of DNA replication. In vitro DNA synthesis with characteristics very similar to DNA replication in vivo is catalyzed by seven highly purified, T4-encoded proteins, and many of the molecular details of the activities of these proteins have been elucidated (C. C. Liu et al., 1979; Alberts et al., 1983; Nossal and Alberts, 1983). This in vitro system is not, however, capable of initiating replication forks at the natural origins of T4 DNA replication. Instead, single-stranded regions or nicks in the DNA template must be provided to bypass the normal mechanism of fork initiation.

The bacteriophage T4-induced type II topoisomerase (L. F. Liu et al., 1979; Stetler et al., 1979) has been implicated in the initiation of T4 replication in vivo, since topoisomerase-deficient mutants display a "DNA-delay" phenotype, characterized by a delayed production of DNA (Mulli and Bernstein, 1974). McCarthy et al. (1976) determined that the rate of replication fork movement in cells infected with these mutants is the same as in cells infected with wild type bacteriophage, and therefore concluded that the process of replication fork initiation is defective. The residual T4 DNA replication seen in topoisomerase-deficient mutants has been reported to be totally dependent on host DNA gyrase, indicating that this host type II DNA topoisomerase can partially substitute for the T4 enzyme (McCarthy, 1979).

As a first step toward reconstructing the replication fork initiation process in vitro, we have characterized the interaction of the T4 topoisomerase with T4 DNA, searching for possible origin-specific DNA recognition by the topoisomerase. In the reaction catalyzed by type II topoisomerases, the enzyme creates a transient break in a double-stranded DNA molecule through which a second segment of unbroken double-stranded DNA is passed (for reviews, see Cozzarelli, 1980; Gellert, 1981). The presumptive reaction intermediate consists of broken DNA with a subunit of the topoisomerase covalently attached to each of the two 5' ends that are newly formed at the break. This intermediate can be detected at a very low frequency after detergent treatment of T4 topoisomerase reactions (L. F. Liu et al., 1979). Studies of a similar intermediate in the DNA gyrase reaction were greatly facilitated by the finding that nalidixic acid and oxolinic acid, two closely related antibacterial agents, trap this intermediate and enable nearly quantitative recovery of the enzyme in the covalent complex (Sugino et al., 1977; Gellert et al., 1977). Oxolinic acid also enhances double-strand DNA breakage by the T4 DNA topoisomerase. Using this drug treatment followed by a new method for isolation of covalent topoisomerase-DNA complexes, we show that both the T4 DNA topoisomerase and Escherichia coli DNA gyrase recognize native T4 DNA in a highly specific fashion.

EXPERIMENTAL PROCEDURES

Materials—Homogeneous T4 topoisomerase was prepared by a modification of the method of L. F. Liu et al. (1979), as described by Kreuzer and Jongeneel (1983). Purified subunits A and B of E. coli DNA gyrase were kindly provided by Dr. N. Cozzarelli (University of California, Berkeley). Restriction enzymes were purchased from New England Biolabs, and T4 DNA polymerase was purified as described (Morris et al., 1979). T4 DNAs were prepared from purified phage particles by gently extracting with neutralized, water-saturated phenol and then ether, followed by extensive dialysis into 10 mM Tris-Cl (pH 7.8), 1 mM Na₂EDTA. HMC glu T4 DNA was purified

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1 The abbreviations used are: HMC glu T4 DNA, native T4 DNA with the normal hydroxymethylated and glucosylated cytosine residues; C T4 DNA, T1 DNA (prepared from a multiple mutant) containing unmodified cytosine residues; HMC T4 DNA, T4 DNA containing nonglucosylated hydroxymethylcytosine residues; kb, kilobase pairs; SDS, sodium dodecyl sulfate; ATPγS, adenosine 5'-O-(thiotriphosphate).
from wild type phage particles; HMC T4 DNA was purified from T4 oxy ggt phage grown in E. coli K803 (both kindly provided by Dr. J. Childs, Chalk River Nuclear Laboratory, Ontario, Canada); and C T4 DNA was purified from T4 56 + 42 denb alk phage particles grown in E. coli ED8689. The authenticity of these DNAs was verified by restriction enzyme analysis; all three were cleaved by TspQ, only C T4 DNA and HMC T4 DNA were cleaved by XbaI, and only C T4 DNA was cleaved by XhoI. Oxolinic acid was kindly provided by Martin Block of Warner-Lambert Co., [α-32P]dTTTP was purchased from New England Nuclear and Amersham, agaro (medium electroendosmosis) was from SeaKem, glass fiber filters (GF/C and GF/A) were from Whatman, and human serum albumin was from Worthington Biochemicals.

Topoisomerase Cleavage Reactions—Except where indicated, all T4 topoisomerase and E. coli DNA gyrase cleavage reactions (20 μl) contained 40 mM Tris-Cl (pH 7.8), 60 mM KCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM Na3EDTA, 0.5 mM ATP, human serum albumin at 30 μg/ml and oxolinic acid at 500 μg/ml. The reactions were incubated for 15 min at 30 °C, and then SDS was added to 0.2%. When necessary, the covalent attachment of the topoisomerase to DNA was eliminated by incubation for 60 min at 37 °C with proteinase K (EM Biochemicals) at 100 μg/ml.

Preparation of End-labeled T4 DNA—DNA fragments were analyzed on horizontal agarose gels in TBE buffer (90 mM Tris base, 90 mM boric acid, 2.5 mM Na3EDTA). Samples were loaded in final concentrations of 4% Ficoll and 1% SDS, with 0.1% bromphenol blue and xylene cyanole added as tracking dyes. After electrophoresis, agarose gels were dried onto filter paper, and autoradiography was carried out at -70 °C with Cronex Lightning Plus intensifying screens.

Spin Dialysis—A rapid method of gel filtration, called spin dialysis, is based on the original method of Neal and Florini (1973) which was modified by McGhee and von Hippel (1977). Spin dialysis columns were prepared by delivering 0.8 ml of a preequilibrated slurry (60% molecular weight materials tie) of siliconized glass beads (average size = 1 mm) to a 1.5-ml Eppendorf tube with a hole (made with a 27-gauge needle) punched in the bottom. The gel matrix (but not the liquid) was retained in this tube by covering the hole with a small amount (30 μl) of siliconized glass beads (average diameter = 0.1 mm) before adding the slurry. The tube was placed into a second 1.5-ml Eppendorf tube (with a hole punched in the side to relieve pressure which develops during centrifugation), and the nested pair of tubes was spun for 2 min at setting 80 in a Clay Adams tabletop centrifuge. This spin removed the gel buffer, and the top tube, containing the packed gel beads, was then nested into an empty 1.5-ml Eppendorf tube. The DNA sample (60 μl) was delivered to the center of the packed column, and the nested tubes were centrifuged as described above. The DNA was recovered from the bottom tube, free of low molecular weight materials (i.e. SDS, unincorporated triphosphates, phenol, salts, etc.). The DNA yield in this procedure was generally 50 to 75%.

Preparation of End-labeled T4 DNA Restriction Digests—The substrates used for Figs. 2, 3, and 6 were end-labeled by replacement synthesis (OFarrell, 1981). After the appropriate restriction enzyme treatment, T4 DNA polymerase was added and exonuclease digestion allowed for 5 min at 37 °C; resynthesis in the presence of [α-32P]dTTP and nonradioactive dCTP, dGTP, and dATP was then used in the detection of covalent complexes. The filter pair was washed five times with 150 μl of binding buffer, and the eluate from the top filter was then analyzed as described above. This procedure detects both covalent and noncovalent topoisomerase-DNA complexes.

RESULTS

Oxolinic Acid Induces DNA Cleavage by the T4 Topoisomerase—As discussed above, an intermediate in the reaction of type II DNA topoisomerases consists of broken DNA with a native molecular weight. In order to facilitate further analysis of the cleavage reaction catalyzed by the T4 topoisomerase, conditions were sought that increase the efficiency of cleavage (and presumably the lifetime of the reaction intermediate). It had previously been shown that nalidixic acid and its more potent analogue oxolinic acid are specific inhibitors of E. coli DNA gyrase and that their addition causes nearly quantitative cleavage of the DNA double helix by the enzyme (Sugino et al., 1977; Gellert et al., 1977). While we detect no effect of oxolinic acid on the T4 topoisomerase at a drug concentration that inhibits E. coli DNA gyrase (10 μg/ml), the drug blocks the T4 topoisomerase at higher concentrations, with half-maximal inhibition at roughly 250 μg/ml (L. F. Liu et al., 1979; data not shown). In the presence of 500 μg/ml oxolinic acid, the T4 topoisomerase converts circular duplex φX174 DNA into a series of linear fragments when SDS is added, as judged by gel electrophoresis. Quantitation of this cleavage by scanning densitometry shows that the amount of DNA cleavage obtained is about 100-fold greater than that caused by SDS addition alone (Fig. 1). Assuming that the native molecular weight of the topoisomerase is 260,000 (see Kreuzer and Jongeneel, 1983), an equimolar ratio of DNA and protein was reached when about 20 ng of enzyme was added in this experiment, and thus the data in Fig. 1 indicate that virtually every topoisomerase molecule causes a

FIG. 1. Oxolinic acid induces DNA cleavage by T4 topoisomerase. Topoisomerase reactions contained 0.3 μg of linear φX174 duplex DNA, the indicated amount of topoisomerase, and either no oxolinic acid (O) or 500 μg/ml oxolinic acid (●). After 10 min at 30 °C, the reactions were terminated by adding SDS, and then treated with proteinase K as described under "Experimental Procedures." The reaction products were separated on a 0.8% agarose gel, and the disappearance of the linear duplex DNA was quantitated by scanning a negative of the ethidium bromide-stained gel, using a Zeineh soft laser densitometer.
DNA cleavage in the presence of oxolinic acid. Both the efficient oxolinic acid-induced cleavage of duplex φX174 DNA and the low level of cleavage seen in the absence of the drug require treatment with a detergent such as SDS to trap the DNA in its broken form, and a prior treatment with excess EDTA blocks DNA cleavage. In addition, in either the presence or absence of oxolinic acid, protein becomes covalently attached to the broken DNA as judged both by filter-binding experiments and comparison of the rate of electrophoretic migration of the DNA with and without proteinase K treatment (data not shown).

Filter-binding Method for Identification of Strong Topoisomerase Cleavage Sites on the T4 Genome—The analysis of T4 topoisomerase-binding sites on T4 DNA is complicated by the fact that native T4 DNA contains glucosylated hydroxymethylcytosine residues, which makes it refractory to nearly all restriction enzymes. Moreover, the T4 genome is large (165 kb), and the chromosome sequence is circularly permuted. A third complication is that the T4 topoisomerase binds to a large number of sites, many with a low efficiency (see below).

We began our analysis by using radioactively labeled restriction enzyme digests of C T4 DNA, prepared from a special multiple mutant bacteriophage (O'Farrell et al., 1980). The restriction enzyme digest was treated with topoisomerase in the presence of oxolinic acid, and the reaction was terminated with SDS to create covalent complexes of topoisomerase and cleaved DNA. A strong cleavage site on the DNA, being located a unique distance from each of the two ends of a DNA restriction fragment, should produce two discretely sized fragments of DNA over a background of DNA fragments created at weaker cleavage sites. To detect such strong sites, the topoisomerase-cleaved DNA was purified away from the substrate DNA restriction fragments by a modified glass fiber filter-binding technique that selects only DNA molecules that have covalently bound protein (see “Experimental Procedures”).

Fig. 2 compares the DNA fragments in a 32P-labeled XhoI digest of C T4 DNA (lane 1) with the DNA fragments obtained by the above filter-binding method either without or with T4 topoisomerase treatment (lanes 2 and 3, respectively). As shown by a comparison of lanes 2 and 3, only DNA fragments with bound topoisomerase stick to the filters. About 30 discrete radioactive bands were created by topoisomerase cleavage, in addition to a background smear of nonspecific cleavage products. Thus, T4 topoisomerase recognizes on the order of 15 high affinity cleavage sites on the C T4 DNA (30 divided by 2, since each cleavage generates two fragments), in addition to a much larger number of lower affinity sites. The addition of ATP to the enzyme reaction had essentially no effect on the pattern of fragments produced, although it did slightly increase the total frequency of cleavage (Fig. 2, lane 4). Oxolinic acid could be added either at the start of the reaction (lane 4) or 10 s before terminating the reaction with SDS (lane 5), without changing the cleavage products obtained. This provides some evidence that the oxolinic acid does not change the distribution of sites to which the enzyme is bound.

Topoisomerase Recognition of Native T4 DNA—Since the mutant T4 cytosine-containing phages are seriously disturbed with respect to various aspects of nucleic acid metabolism in vivo, it seemed imperative to compare the topoisomerase recognition of T4 cytosine-containing DNA with that of the native, glucosylated hydroxymethylcytosine-containing DNA. As mentioned above, native T4 DNA is, in general, refractory to restriction enzyme cleavage. However, after screening large number of restriction enzymes, we discovered that TaqI is unusual in cleaving native T4 DNA efficiently. The TaqI restriction digests of C and HMC glu T4 DNA are compared in lane 1 of Fig. 3, A and B, respectively. Many of the faster migrating bands on the gel contain several co-migrating restriction fragments, and we estimate that more than 100 TaqI sites are present in T4 DNA (data not shown).

There are differences in the pattern of DNA fragments obtained by TaqI digestion of C and HMC glu T4 DNAs. These differences arise from at least three separate causes: 1) the C T4 DNA contains a deletion removing the 162.3-165.5-kb region of the T4 genome; 2) HMC glu T4 DNA has a few TaqI sites that are protected from restriction enzyme cutting; and 3) the glucose moieties change the electrophoretic migration of the HMC glu T4 DNA fragments. Because of these

\[ \text{Site Specificity of T4 Topoisomerase on T4 DNA} \]

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3 D. Coit, unpublished data.

4 C. V. Jongeneel, unpublished data.
differences in the pattern of fragments, it is not possible to identify the same DNA fragment in the two digests with certainty simply on the basis of electrophoretic migration. Nevertheless, the topoisomerase cleavage sites on these two DNAs can be compared both qualitatively and quantitatively.

In the absence of ATP, several strong cleavage fragments (together with numerous weaker ones) were generated by the T4 topoisomerase using the TaqI-digested C T4 DNA substrate (Fig. 3A, lanes 3 and 4). At the highest enzyme level, a smear of low molecular weight fragments was generated (lane 5), indicating that many of the TaqI restriction fragments were cleaved more than once by the topoisomerase. The addition of ATP had little or no effect on the particular fragments recovered, but it did increase the efficiency of cleavage by about 2-fold (lanes 6-8). These results are consistent with those obtained above using XhoI-digested C T4 DNA as substrate, and demonstrate that the T4 topoisomerase recognizes a large number of cleavage sites on C T4 DNA.

The results of the cleavage site assay with TaqI-digested HMC glu T4 DNA were quite different (Fig. 3B). In the absence of ATP, even at the highest enzyme level (Fig. 3B, lane 5), only about eight strong and eight weaker cleavage fragments were generated, and the pattern was not obscured by multiple cutting of the larger fragments. Thus, in the absence of ATP, the T4 topoisomerase shows a marked increase in specificity when recognizing HMC glu T4 DNA as compared to C T4 DNA. Because DNA fragments generated from C T4 DNA and HMC glu T4 DNA substrates are not directly comparable with respect to their electrophoretic migration, it is not possible at this time to assess whether some of the same strong topoisomerase cleavage sites are recognized on the two DNAs.

The addition of ATP increased the frequency of topoisomerase-induced cleavage of HMC glu T4 DNA roughly 5-fold (Fig. 3B, lanes 6-8). At the highest enzyme level, multiple cutting of the largest DNA cleavage fragments was observed, but about 12 to 14 specific cleavage fragments were still evident (lane 8). Note that the strong cleavage fragments generated in the absence of ATP are also seen at the lower enzyme levels in the presence of ATP, and therefore, the same strong recognition sites were generally recognized in both cases. The stimulatory effect of ATP could be either a simple quantitative effect on the efficiency of cleavage, or alternatively, indicate that numerous weaker recognition sites will react only in the presence of the cofactor.

Since the activity of the topoisomerase in the strand-passing reaction is coupled to ATP hydrolysis (L. F. Liu, et al., 1979), the ATP-induced increase in the efficiency of topoisomerase cleavage of HMC glu T4 DNA could involve some ATP hydrolysis-dependent reaction of the topoisomerase, such as localized supercoiling or movement of the enzyme along the DNA double helix. This hypothesis was tested by...
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determining whether the nonhydrolyzable ATP analogue ATP•S would mimic the effect of ATP; this analogue is a potent inhibitor that blocks turnover of the enzyme (L. F. Liu et al., 1979). As shown in Fig. 3B (lane 9), ATP•S closely mimics ATP in its effect on topoisomerase-induced DNA cleavage. Thus, it seems likely that the ATP effect shown in Fig. 3B simply reflects a conformational change of the enzyme caused by ATP binding (see below).

The increased specificity with which the topoisomerase recognizes native T4 DNA could require either the hydroxymethylation of the cytosine residues or the glucosylation of these hydroxymethylcytosines. These two possibilities were distinguished by preparing 32P-labeled TaqI restriction fragments of hydroxymethylated T4 DNA free of glucose residues, which was isolated from an ogt, lgt double mutant T4 bacteriophage (Revel and Luria, 1970). The topoisomerase-induced cleavage patterns generated from the glucose-free DNA (Fig. 3C) were nearly identical with those seen with C T4 DNA (Fig. 3A). Moreover, the dramatic stimulation of cleavage seen with native T4 DNA in the presence of ATP (Fig. 3B) was not obtained with the glucose-free DNA. We therefore conclude that glucosylation causes the increase in the specificity of topoisomerase recognition of native T4 DNA.

Since E. coli DNA gyrase has been reported to substitute partially for the T4 topoisomerase during DNA replication in vivo (McCarthy, 1979), we compared its cleavage specificity with that of the T4 topoisomerase on T4 DNA substrates. With HMC glu T4 DNA, only about 10 major cleavage fragments were observed, indicating that gyrase recognizes approximately five strong cleavage sites on this substrate, and the background of nonspecific cleavage was lower than for the T4 topoisomerase at comparable levels of DNA cleavage (compare Fig. 4, lane 3 with lane 1). The pattern of cleavage fragments generated after gyrase treatment of C T4 DNA also showed about 10 strong cleavage fragments and a low background of nonspecific cleavage fragments (data not shown). The addition of ATP to the gyrase reactions had essentially no effect on the pattern of cleavage fragments observed (Fig. 4, lane 4). Some of the DNA gyrase-induced cleavage fragments co-migrated with the T4 topoisomerase-induced fragments, but our preliminary mapping analysis indicates that these cleavage fragments originate from different regions of the T4 genome.

One possible limitation of the filter-binding assay used here is that the topoisomerases might not bind to some possible recognition sites, due to their location close to the end of a DNA fragment (i.e. near a TaqI restriction site). This was tested by repeating the filter-binding experiment in such a way that the cleavage fragments were generated by topoisomerase on intact T4 DNA, with the TaqI digestion only performed after the topoisomerase reaction (Fig. 4, lanes 5–8). Except for relatively minor differences in intensities, the recognition sites for T4 topoisomerase in the absence (lanes 1 and 5) or presence of ATP (lanes 2 and 6), and for DNA gyrase in the absence (lanes 3 and 7) or presence of ATP (lanes 4 and 8), were the same on intact and on TaqI-cleaved T4 DNA. Thus, the spectrum of cleavage fragments detected is not significantly affected by DNA substrate size.

The high level of specificity demonstrated for the T4 topoisomerase and the E. coli DNA gyrase on native T4 DNA could, in principle, be explained by either of the following situations: 1) The initial binding of the enzyme occurs at strongly preferential sites on the DNA, and all of these sites undergo a strand-scission reaction that results in a covalent complex. 2) Only selected DNA binding sites are revealed by the covalent complex. For example, topoisomerase action, involving cleavage of the phosphodiester backbone with concomitant formation of the covalent protein-DNA complex, might occur at only a subset of the enzyme-binding sites.

The above two possibilities were distinguished by comparing the DNA fragments retained in two different filter-binding assays. For detecting both covalent and noncovalent complexes, 32P-labeled TaqI restriction fragments of native T4 DNA were incubated with various levels of T4 topoisomerase in the presence of oxolinic acid, and half of each reaction was applied without SDS treatment to a GF/C filter; the bound DNA was then eluted with SDS and analyzed (Fig. 5A, lanes 2 through 5). This filtration should select both noncovalent complexes (in which the restriction fragment substrate would not be cleaved by the topoisomerase), and covalent complexes (where the substrate would be cleaved into two subfragments upon SDS treatment of the DNA on the GF/C filter). To detect only covalent complexes, the other half of each reaction was treated with SDS and subjected to the filter-binding assay (Fig. 5B, lanes 2 through 5). In the assay that detects both covalent and noncovalent DNA binding, the enzyme bound
to at least one site on virtually every *TaqI* restriction fragment, and no strong site specificity was seen even after dilution of the enzyme. Because most of the DNA fragments recovered co-migrated with the original *TaqI* restriction fragments, most of the enzyme-bound DNA was apparently not cleaved by the topoisomerase. In contrast, the usual pattern of specific topoisomerase-cleaved DNA fragments was observed in the assay that selects for only covalent complexes. We conclude that only a small subset of the initial binding sites can produce a covalent complex. Presumably, this reflects the fact that most binding sites on HMC glu T4 DNA are unable to undergo the topoisomerase-induced, reversible strand breakage event under these conditions (see "Discussion" below).

A markedly different result is obtained when the same type of comparison is made for DNA gyrase binding to native T4 DNA. Here, essentially the same specific DNA fragments are recovered in the two DNA-binding assays (Fig. 5, A and B, lane 6). Thus, the site specificity of DNA gyrase on native T4 DNA results directly from the initial selection of binding sites, and there is no evidence for a class of binding sites that are unable to undergo the strand cleavage reaction.

As shown in Fig. 1, the addition of oxolinic acid to a reaction containing a DNA substrate with unmodified cytosine residues causes nearly every T4 DNA topoisomerase molecule to cleave a duplex DNA molecule once SDS is added. In contrast, only a minority of the enzyme molecules cleave modified HMC glu T4 DNA under the same conditions (Fig. 5). We find that oxolinic acid stimulates cleavage of HMC glu T4 DNA by roughly 20-fold in the absence of ATP, and nearly 100-fold in the presence of the cofactor (data not shown). The patterns of specific DNA cleavage products obtained when SDS is added to reactions without (Fig. 6) or with oxolinic acid (Fig. 3B) are significantly different. (Some of the fragments recovered in the two experiments have the same electrophoretic mobility, but further analysis is necessary to determine if these fragments are identical.) Another difference is that there is no apparent effect of ATP on the cleavage observed in the absence of oxolinic acid (Fig. 6, lanes 3 and 4). The cleavage sites observed with and without oxolinic acid apparently represent different classes of binding sites on native T4 DNA (see "Discussion" below).

**DISCUSSION**

The T4 DNA topoisomerase and *E. coli* DNA gyrase share several properties, including the double strand passage mechanism of topoisomerization, a multisubunit composition, and an ATP hydrolysis requirement (L. F. Liu *et al.*, 1979; L. F. Liu *et al.*, 1980). The results presented here extend the similarities between the two enzymes. Oxolinic acid, a potent inhibitor of DNA gyrase, also blocks T4 topoisomerase, albeit...
at higher drug concentrations. For both enzymes, the drug appears to interfere with resealing of the transient reaction intermediate consisting of a DNA helix broken in both strands with the enzyme covalently attached. In addition, both enzymes cleave a broad spectrum of sites with widely varying efficiencies in φX174 duplex DNA, and if very weak cleavage sites are counted, both recognize roughly one cleavage site every 50 base pairs. A detailed analysis comparing sites in limited regions of the genome suggests that about one-fourth of the cleavage sites in φX174 duplex DNA are recognized in common by the two enzymes, but the relative efficiencies of cleavage at commonly recognized sites are very different for the two enzymes. The rules of DNA sequence recognition by type II topoisomerases are elusive (Morrison and Cozzarelli, 1979), but a detailed comparison of the sequences cleaved by these two related enzymes could help to define these rules.

The filter-binding assay introduced here uses SDS treatment to destroy all noncovalent protein interactions with DNA, followed by a rapid method of gel filtration called "spin dialysis" to remove both the SDS and the noncovalently bound protein. It provides an effective method for selectively purifying covalent protein-DNA complexes, and is so sensitive that covalent complexes of topoisomerase bound at specific locations on exogenously added, native T4 DNA can be detected even if the source of topoisomerase is a crude extract of T4-infected cells. The pattern of cleavage fragments and the effect of ATP in these extracts are the same as described for the purified topoisomerase, suggesting that no elements that alter topoisomerase specificity are lost during topoisomerase purification. This assay should prove useful in studying the binding of any protein that interacts covalently with DNA.

The efficiency with which duplex DNA is cleaved by the T4 DNA topoisomerase is not only strongly enhanced by the inhibitor oxolinic acid, but also influenced by the presence of ATP and the modification of cytosine residues in the substrate DNA. With an unmodified substrate, such as duplex φX174 DNA, oxolinic acid increases the efficiency of cleavage by about 100-fold (Fig. 1), and there is no dramatic effect of ATP on DNA cleavage. In this case, oxolinic acid appears to very effectively trap the reaction intermediate consisting of broken duplex DNA covalently attached to the topoisomerase, since virtually every enzyme molecule cleaves a duplex DNA substrate molecule in the presence of the inhibitor. The cleavage of unmodified (cytosine-containing) T4 DNA by the T4 topoisomerase seems quite similar. However, the efficiency with which the T4 topoisomerase cleaves native HMC glu T4 DNA in the absence of ATP is greatly reduced. As judged by a comparison of the DNA fragments recovered in the noncovalent and the covalent binding assays (Fig. 5), only a subset of the initial binding sites on native T4 DNA is cleaved by the topoisomerase in the presence of oxolinic acid. Interestingly, the addition of ATP dramatically increases the frequency of cleavage of this modified DNA substrate (Fig. 3B). The fact that this stimulation is also caused by the nonhydrolyzable analogue ATPγS suggests that it is not caused either by ATP-induced movement of the enzyme to new cleavage sites or by an induction of local DNA supercoiling. Instead, it seems likely that the ATP stimulation reflects a conformational change of the enzyme induced by the binding of ATP or ATPγS. Analogously, an ATP-induced shift in DNA gyrase cleavage sites on Co/EI DNA has been shown to depend on a conformational change induced by ATP binding (Morrison et al., 1980).

For both DNA gyrase and the T4 topoisomerase, binding of ATP (or a nonhydrolyzable ATP analogue) apparently induces a conformational change that allows a single breakage-reunion reaction cycle, and the actual hydrolysis of ATP is required for reuse of the enzyme in a second reaction cycle (Singo et al., 1978; L. F. Liu et al., 1979). One might therefore expect that both enzymes would require the binding of ATP to induce double strand cleavage. In this view, the considerable ATP dependence shown for the cleavage of native T4 DNA by the T4 topoisomerase is what one would naively expect in all cases. Why is there a tighter coupling to ATP when the T4 topoisomerase acts on its native substrate than when it acts on T4 DNA lacking glucosyl modifications (Fig. 3)? Could the topoisomerization reactions catalyzed by the T4 topoisomerase likewise be different in the presence of the modified cytosine residues? Previous studies revealed that the enzyme is unable to actively supercoil circular duplex DNA substrates containing unmodified cytosine residues (Liu et al., 1979; Stetter et al., 1979), but it seems important to retest for possible DNA supercoiling using covalently closed duplex DNA circles constructed from native (HMC glu) T4 DNA.

The oxolinic acid-enhanced cleavage of native T4 DNA by the T4 DNA topoisomerase occurs at strongly preferred sites (Fig. 3B). In general, the same preferred sites are cleaved in the presence or absence of ATP, even though the efficiency of cleavage is increased by the presence of the cofactor. We cannot be certain that these strong cleavage sites represent the strongest binding sites of the enzyme on T4 DNA, since only a minority of the enzyme molecules generate a covalent DNA-protein complex (Fig. 5). However, we are unable to detect any strong sites that do not result in cleavage, because no specific T4.1 restriction fragments are recovered preferentially upon dilution of the enzyme in an experiment that detects noncovalent DNA binding (Fig. 5A).

The findings that ATP has no effect on the cleavage reaction and that the preferred sites of DNA cleavage are different in the absence of oxolinic acid suggest that the cleavage that occurs in the absence of oxolinic acid may occur at a special subset of topoisomerization sites where the reformation of the intact DNA double helix from the covalent reaction intermediate is unusually slow. In this case, the strong cleavage sites observed in the presence of oxolinic acid would be more indicative of the strong sites of T4 topoisomerase action. Further experiments are in progress to test this supposition directly.

The mechanism of initiation of T4 DNA replication in vivo has been the object of intensive study. Several groups have shown that T4 DNA replication normally begins at preferred regions of the T4 chromosome; these "replication origins" have been approximately localized by hybridizing newly synthesized DNA to defined fragments of cytosine-containing T4 DNA (Halpern et al., 1975; Mosig et al., 1981; King and Huang, 1982). Differences in the locations of the origins detected in different laboratories have recently been explained as being primarily due to physiological perturbations induced by the particular experimental protocols used by the various groups, and it now seems that T4 can utilize as many as five different replication origins depending on the conditions (Mosig, 1983). Recently, it has also been shown that T4 replication fork initiation occurs in two separate modes (Luder and Mosig, 1982). The initial mode, which produces replication intermediates consisting of standard "replication bubbles," requires a direct involvement of the host RNA polymerase, while the later mode is independent of RNA polymerase but

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*K. N. Kreuzer, unpublished data.

*C. F. Morris and M. Bittner, personal communication.*
dependent on phage-encoded genetic recombination enzymes. The RNA polymerase-dependent mode functions at an origin located roughly in the 15–20-kb region of the T4 chromosome, but it may also operate at other origins. In addition, at least three of the origin regions correlate with locations of recombinational hot spots, and these hot spots may result in preferred initiation in these regions by the second mode described above.

Mosig’s group has also obtained evidence for a role of the T4 DNA topoisomerase in both modes of replication fork initiation (Mosig, 1983). In the experiments presented in this paper, we find that the T4 topoisomerase acts preferentially at a small number of sites on native T4 DNA. The preliminary results of genome-mapping experiments have been published (Alberts et al., 1983). One strong oxolinic acid-induced topoisomerase cleavage site is located near the strongest early T4 promoter (Niggemann et al., 1981), in the vicinity of a replication origin that functions in the RNA polymerase-dependent mode. Another strong cleavage site is located near a major recombinational hot spot on the T4 chromosome, near or within gene 35. Recombination promoted at this hot spot is dependent on glucosylation of the participating DNAs (Levy and Goldberg, 1980), which is reminiscent of our finding that topoisomerase recognition of T4 DNA is grossly altered by glucosylation of the DNA. Further experiments will be necessary to determine whether the topoisomerase site specificity studied in this report is involved in the selection and utilization of T4 replication origins by one or both of the two different modes of replication fork initiation.

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