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Role of DNA gyrase subunits in synthesis of bacteriophage φX174 viral DNA

(*in vitro* DNA synthesis/nalidixic acid/novobiocin/topoisomerase II')

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ABSTRACT **The role of *Escherichia coli* DNA gyrase subunit A and subunit B during φX174 viral DNA synthesis was investigated. Addition of nalidixic acid (an inhibitor of gyrase subunit A) and novobiocin (an inhibitor of gyrase subunit B) to an *in vitro* system capable of synthesizing φX174 viral DNA inhibited DNA synthesis. The inhibition caused by novobiocin, however, was not due specifically to an inhibition of gyrase subunit B because DNA synthesis in an *in vitro* system composed of an extract containing novobiocin-resistant gyrase subunit B was also inhibited by novobiocin. The requirement for gyrase subunit A and the dispensability of gyrase subunit B during viral strand synthesis was confirmed *in vivo* by examining φX174 viral DNA synthesis in host bacteria containing temperature-sensitive gyrase subunits.**

Bacteriophage φX174 undergoes three stages of DNA synthesis during infection of *Escherichia coli* (for review, see ref. 1). Stage I DNA synthesis is the conversion of the circular single-stranded viral DNA to double-stranded replicative form (RF) DNA. No viral proteins are required for this conversion. The host replicative machinery usurped by φX174 for stage I synthesis is thought to be that used for lagging strand synthesis on the host chromosome (2). Stage II DNA synthesis is the production of daughter RF molecules. In addition to the proteins required for stage I synthesis, host rep protein (3) and DNA gyrase (4), an enzyme that introduces superhelical turns into closed circular DNA (ref. 5; for review, see ref. 6) are required for stage II synthesis. The only phage-encoded protein required for stage II synthesis is the gene A protein (7) which initiates replication by nicking supercoiled RF DNA at the origin (8, 9). Stage III DNA synthesis is the synthesis of single-stranded viral DNA and requires the phage-coded *A*, *B*, *C*, *D*, *F*, *G*, and *H* gene products (for review, see ref. 10). The host enzymes involved in stage III DNA synthesis are not as well defined as the phage-encoded proteins but probably include most of those involved in stage II DNA synthesis. We have investigated the role of one of these host enzymes, DNA gyrase, during stage III DNA synthesis.

Two methods were used in this investigation: (*i*) gyrase inhibitors were added to an *in vitro* DNA synthesizing system developed by us that is specific for stage III DNA synthesis (11–13); and (*ii*) temperature-sensitive (*ts*) DNA gyrase mutants were infected at the permissive temperature and were shifted to the nonpermissive temperature after stage I and stage II of DNA synthesis were allowed to occur. The DNA synthesized at the non-permissive temperature was labeled and analyzed by sucrose gradient centrifugation.

Using these two methods we have found that gyrase is not involved in stage III DNA synthesis because gyrase subunit B (Gyr B) is not essential for viral strand synthesis. Gyrase subunit A (Gyr A), however, is necessary and its possible role in viral and bacterial replication is discussed.

MATERIALS AND METHODS

Bacteria and Phage Strains. *E. coli* HF4704 (14) and H570–22 (11) have been described. RKH201 is a spontaneous coumermycin-resistant derivative of H570–22. RKH301 is a spontaneous nalidixic acid (Nal)-resistant derivative of H570–22. N4177 is from M. Gellert and contains a temperature-sensitive and coumermycin-resistant *gyrB* gene product (M. Gellert, personal communication). This was moved by P1 transduction into an *ilv*⁻ derivative of HF4704 to make RKH211. KNK453, which is HF4704 with a temperature-sensitive *gyrA* gene product, was from N. Cozzarelli (15). φX174 N11 contains an amber mutation in the *E* gene (lysis gene). φX174 och11am3 (ochre mutation in gene *B* and amber mutation in gene *E*) were from F. D. Funk.

Reagents and Media. Mitomycin C and the sodium salts of Nal and novobiocin (Novo) were from Sigma. Coumermycin A_1 was from D. Smith and J. Davies. RF I DNA was isolated as described (16) except that ethidium bromide/CsCl centrifugation was substituted for the MAK column. RF IV DNA (relaxed, closed circular, RF DNA) was prepared from RF I by treatment with chicken nicking-closing enzyme obtained from E. P. Geiduschek. Low-salt HFB was 30 mM Tris·HCl, pH 7.4/8.6 mM NaCl/40 mM KCl/ 7.6 mM ammonium sulfate/1 mM $MgCl_2$/1 mM $CaCl_2$. Low-salt HFC was 10 mM sodium phosphate, pH 7.4/0.5% glucose/0.08% sodium pyruvate containing each of the 20 amino acids at 50 μg/ml and thymine at 2 μg/ml, both in low-salt HFB.

Gyrase Supertwisting Assay. The sensitivity of gyrase supertwisting activity to Novo was assayed on partially purified DNA gyrase subunits resolved by DEAE-cellulose column chromatography as described (15). Gyrase assays (15) contained 23 fmol of φX174 RF IV DNA. One unit of gyrase subunit gave maximal supertwisting activity in the presence of an excess of the other subunit. The reactions (total volume, 17 μl) were stopped after 30 min at 30°C by addition of 5 μl of 5% NaDodSO$_4$/25% (vol/vol) glycerol containing bromophenol blue at 0.25 mg/ml. Samples were loaded on 13 × 15 × 0.2 cm slab gels of 1% agarose in 40 mM Tris·HCl, pH 7.2/20 mM sodium acetate/1 mM EDTA. Electrophoresis was for 3 hr at 80 V. Gels were stained in ethidium bromide (1 μg/ml) for 15 min and photographed under ultraviolet light.

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Abbreviations: RF, replicative form; Gyr A, gyrase subunit A; Gyr B, gyrase subunit B; Nal, nalidixic acid; Novo, novobiocin; *ts*, temperature-sensitive.

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***In Vivo* DNA Labeling.** Cells (40 ml) were grown to OD_{660} of 0.5 at 30°C in low-salt HFC medium, resuspended in 0.5 vol of low-salt HFB, and treated with mitomycin (50 μg/ml) for 20 min at 30°C. The cells were centrifuged at room temperature and the cell pellet was resuspended in 2.5 ml of low-salt HFB containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$. φX174 N11 phage was added at a multiplicity of infection of 10. After a 5-min adsorption period at 30°C, the infected cells were added to 17.5 ml low-salt HFC at 30°C to begin the infection. A 2-ml aliquot was transferred, at 0 min and at 15 min after infection, to 8.0 ml of low-salt HFC prewarmed to 42°C. The infected cells were aerated by vigorous rotation in a New Brunswick gyratory water bath shaker. Cells were labeled with [^{3}H]thymidine (5 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) at 60 min after infection for a 2-min period. Labeling was terminated by pouring the culture onto 5 ml of frozen HFB buffer. Cell extracts were isolated as described (17) except that RNase T_1 (30 μg/ml) was added during lysis and $MgCl_2$ was omitted during the heating step.

RESULTS

Effect of DNA Gyrase Inhibitors on *in Vitro* Stage III DNA Synthesis. Our *in vitro* system is composed of an extract of cells infected with φX174 gene *B* mutant phage supplemented with purified φX174 proheads. Various *B* mutant-infected cell extracts were obtained for analysis by infecting host cells differing in their sensitivity to gyrase inhibitors. H570-22 is sensitive to both Nal [an inhibitor of Gyr A (18, 19)] and coumermycin. Coumermycin and the structurally related Novo (20) both inhibit Gyr B (21). The infected cell extract made from this strain will be referred to as drug-sensitive extract. RKH201 is resistant to coumermycin and RKH301 is resistant to Nal. Infected cell extracts made from these strains will be referred to as drug-resistant extracts.

The lesion in the phage *B* gene prevents the formation of phage proheads and thus blocks stage III DNA synthesis (13). When the *B* mutant cell extract is complemented *in vitro* by the addition of phage proheads, it is capable of incorporating deoxyribonucleoside triphosphates into DNA of phage particles and intermediates of phage morphogenesis (13). These products may be analyzed by sedimentation on sucrose gradients. Typical patterns of synthesis in the presence of intermediate levels of gyrase inhibitors are shown in Fig. 1. The fastest sedimenting peak of DNA represents viral DNA that has been packaged into phage capsids. The middle peak of DNA sedimenting with an S value of about 50 is double-stranded RF DNA with a single-stranded tail (rolling circle or σ DNA). This DNA is complexed to phage capsid proteins (17, 22) and presumably the viral and host replicative enzymes. Elongation, packaging, and terminating synthesis of viral DNA occur in this complex (17, 22). The slowest sedimenting peak of DNA is RF II DNA (RF DNA with a nick in one strand). This DNA is labeled in the (+) strand only (11) and represents template DNA that has already undergone at least one round of replication.

Nal caused a significant decrease in the counts incorporated into phage particles of the drug-sensitive extract (Fig. 1*a*) in comparison with the Nal-resistant extract (Fig. 1*b*). The pattern of counts for the Nal-resistant extract in the presence of Nal was identical to that in the absence of Nal except for a slight depression in phage synthesis. In contrast, Novo affected both the drug-sensitive and coumermycin-resistant extract to about the same extent (Fig. 1 *c* and *d*).

These results are more readily seen in dose–response curves. Nal inhibited the formation of phage particles (Fig. 2*a*). That this was due to inhibition of Gyr A is suggested by the lack of inhibition in the Nal-resistant cell extract (Fig. 2*b*). Novo also inhibited phage particle formation as well as incorporation into

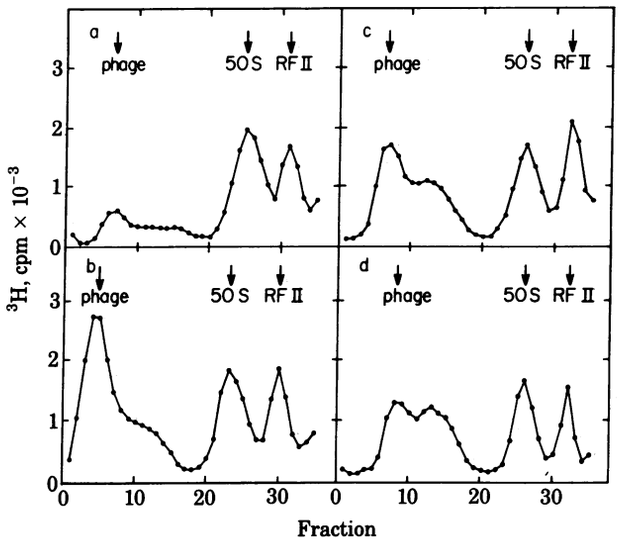

FIG. 1. Inhibition of *in vitro* DNA synthesis by Nal and Novo. *In vitro* DNA synthesis reactions were performed as described (13) using [^{3}H]TTP (200-250 cpm/pmol) to label the DNA. Infected cell extracts were prepared by infecting H570-22, RKH201, and RKH301 with *och1 1am3* φX174 phage (12). Phage proheads were isolated from *och12am3* φX174 phage-infected H570-22 as described (13), concentrated by ammonium sulfate precipitation, dialyzed against 20 mM Tris·HCl, pH 7.2/1 mM $MgCl_2$/50 mM NaCl/1 mM dithiothreitol/10% (wt/vol) sucrose, and stored at −80°C. Prohead (20 μg) was added to 15 μl of phage-infected H570-22 extract (*a* and *c*), to 15 μl of phage-infected RKH301 extract (*b*), and to 15 μl of phage-infected RKH201 extract (*d*). Reaction mixtures contained Nal at 150 μg/ml (*a* and *b*) or Novo at 300 μg/ml (*c* and *d*). Centrifugation on sucrose gradients, performed as described (12), is from right to left.

the 50S complex (Fig. 2*c*) but the Novo dose-response curve for the coumermycin-resistant extract (Fig. 2*d*) was similar to that of the drug-sensitive extract. This suggests that the inhibition of stage III DNA synthesis by Novo was nonspecific rather than due to the requirement for Gyr B activity. The difference in the curves for counts incorporated into the 50S complex in the drug-sensitive and drug-resistant extract (Fig. 2 *c* and *d*) falls within the variability of Novo sensitivity observed in the extracts.

Gyrase Subunits from Coumermycin-Resistant Cells Have Novo-Resistant Supertwisting Activity. A possible explanation for the similar Novo sensitivities of H570-22 and RKH201 in stage III DNA synthesis is that the coumermycin resistance of RKH201 does not reside in the *gyrB* gene. Gyr B from RKH201 would then be wild type and inhibitable by Novo. To test this possibility, gyrase subunits from the wild-type and coumermycin-resistant cells were partially resolved on a DEAE-cellulose column and their sensitivity to Novo was determined by assaying their supertwisting activity. Gyrase supertwisting activity reconstituted from combining the A and B subunits from H570-22 was sensitive to Novo (Fig. 3). The Gyr B from RKH201 had gyrase supertwisting activity that required a 100 times higher Novo concentration than did the wild-type Gyr B in order to inhibit gyrase supertwisting activity. This is the behavior expected of gyrase isolated from strains with coumermycin resistance residing in the *gyrB* gene (21). Thus, the Novo inhibition of stage III DNA synthesis for the drug-sensitive and drug-resistant cell extracts was not due to the specific inhibition of Gyr B.

Gyr A from RKH301 had Nal-resistant supertwisting activity (data not shown). The Nal inhibition of DNA synthesis in the

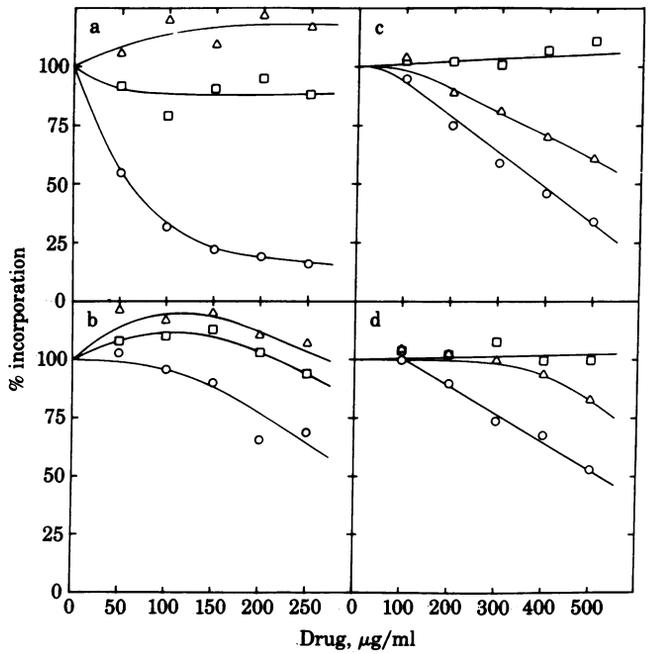

FIG. 2. Dose-response curves of Nal and Novo inhibition of *in vitro* DNA synthesis. Counts incorporated into phage particles (○), 50S complex (△), and RF II DNA (□) of reactions described in Fig. 1 for various drug concentrations were summed and plotted against the corresponding drug concentration. (*a*) Effect of Nal in H570-22 extract; (*b*) effect of Nal in RKH301 extract; (*c*) effect of Novo in H570-22 extract; and (*d*) effect of Novo in RKH201 extract.

drug-sensitive extract is thus due to the specific inhibition of Gyr A.

Gyr A Involvement in Stage III DNA Synthesis *in Vivo*. Our *in vitro* stage III replication system clearly showed that Nal inhibition of Gyr A caused a decrease in viral DNA synthesis. There was the possibility, however, that this was an artifact of Nal binding to gyrase and forming a poison as has been proposed by Kreuzer and Cozzarelli (15) for Nal inhibition of DNA synthesis. Therefore, it was desirable to inhibit Gyr A during viral DNA synthesis by some means other than Nal. This was accomplished by using an *E. coli* strain with a *ts* Gyr A protein (15). KNK453 is isogeneic with HF4704 except for the *ts gyrA* mutation. When shifted to 42°C immediately after infection and labeled during stage III DNA synthesis, *E. coli* HF4704 showed a normal pattern of [^{3}H]thymidine incorporation when the products were analyzed by sucrose gradient centrifugation (Fig. 4*a*). In contrast to this, KNK453 showed little DNA synthesis under the same conditions (Fig. 4*b*). This is explicable from the requirement for supertwisted RF I during transcription (16, 23) and for initiation of stage II replication (4), both of which are prerequisites for stage III replication. When RF I was allowed to accumulate in KNK453 by infecting for 15 min before the shift-up, similar results were obtained (Fig. 4*e*). The reduced level of viral DNA synthesis in the *ts* mutant thus implicates Gyr A in stage III DNA synthesis.

The same experiments were performed with a *ts gyrB* mutant host. Only a small amount of viral DNA was synthesized when the *ts gyrB* host (RKH211) was shifted up immediately after infection (Fig. 4*c*). When RF I was allowed to accumulate, however, there was no effect on stage III DNA synthesis at the nonpermissive temperature (Fig. 4*f*), confirming the *in vitro* observation of the noninvolvement of Gyr B during stage III replication.

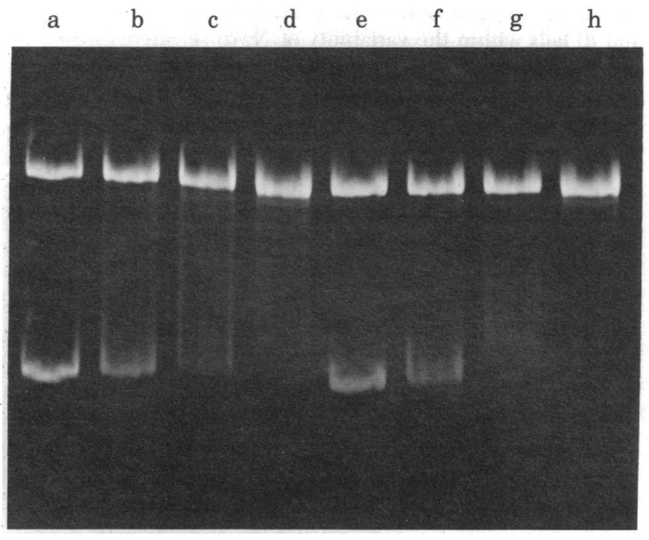

FIG. 3. Novo sensitivity of Gyr B in gyrase supertwisting activity from drug-sensitive and drug-resistant cells. One unit of Gyr A from H570-22 was reconstituted with 1 unit of Gyr B isolated as described (15) from H570-22 (lanes a-d) and 1 unit of Gyr B from RKH201 (lanes e–h). Gyrase assays were performed in the presence of the following concentrations of Novo: lanes a and e, none; b, 0.1 μg/ml; c, 0.3 μg/ml; d, 1 μg/ml; f, 10 μg/ml; g, 30 μg/ml; h, 100 μg/ml.

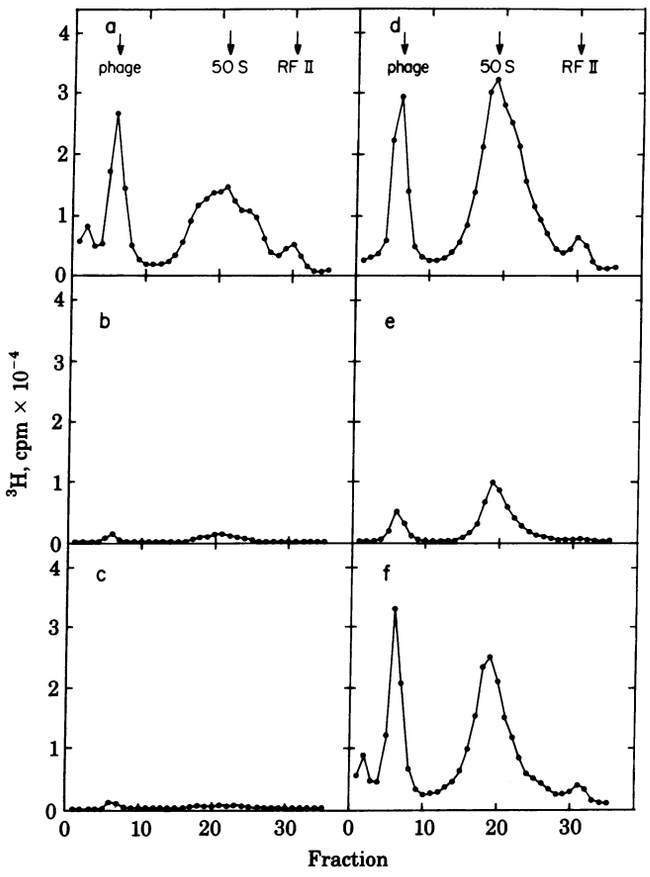

FIG. 4. *In vivo* labeling of φX174 DNA synthesized at 42°C. φX174 N11-infected HF4704 (*a* and *d*), *ts gyrA* KNK453 (*b* and *e*), and *ts gyrB* RKH211 (*c* and *f*) were labeled 60 min after infection, and extracts obtained. Shift-up to 42°C occurred at 0 min after infection (*a*, *b*, and *c*) or at 15 min after infection (*d*, *e*, and *f*). Conditions for sucrose gradient centrifugation were the same as for Fig. 1.

DISCUSSION

The role of DNA gyrase during φX174 stage III DNA synthesis was investigated. We have determined that gyrase itself is not involved because Gyr B is not essential during stage III replication. Evidence for this was obtained from two independent methods of analysis.

(*i*) High concentrations of Novo did not cause a gyrase-dependent inhibition in our *in vitro* DNA synthesizing system. We judged that the inhibition was not gyrase dependent because the extract obtained from coumermycin-resistant cells, containing Novo-resistant Gyr B, was also inhibited in *in vitro* stage III DNA synthesis by Novo. The inhibition caused by Novo may be due to the inhibition of DNA polymerase III since this enzyme is inhibitable by coumermycin (24).

(*ii*) Thermal inactivation of *ts* Gyr B had no effect on stage III replication *in vivo* when supertwisted RF templates were allowed to accumulate. We believe that the difference between the two gyrase mutants in amount of DNA synthesized when the temperature shift occurred after 15 min (Fig. 4 *e* and *f*) reflects the different requirements for the two gyrase subunits during φX174 infection. The difference does not result from the *ts gyrB* mutant being leakier than the *ts gyrA* mutant because, when the temperature shift occurred immediately after infection, the *ts gyrB* mutant had less DNA synthesis than the *ts gyrA* mutant (Fig. 4 *b* and *c*).

On the basis of *in vitro* studies using purified enzymes, Eisenberg *et al.* (8) proposed that, after nicking RF I, the φX174 A protein remains covalently bound to the RF template and is reutilized processively for the cutting and ligation of viral single-stranded DNA during many rounds of replication. We have confirmed a key feature of their model—namely, that, because DNA gyrase is not required during stage III replication, the RF template does not pass through a supertwisted state in order to have a new round of viral strand synthesis.

Although gyrase was not involved in stage III replication, one of its subunits, the Gyr A protein, functioned in some aspect of viral strand synthesis. Inactivation of Gyr A by Nal or thermal inactivation in a *ts* host resulted in the inhibition of viral DNA synthesis. The inhibition of stage III DNA synthesis in the *gyrA* mutant host (Fig. 4*e*) did not result from an absence of phage proteins at the time of labeling. A deficiency of phage proteins could occur if gyrase is needed continuously to maintain a population of supertwisted RF I molecules for transcription of phage RNA. This did not happen because the *ts gyrB* mutant host could support stage III DNA synthesis under the same conditions (Fig. 4*f*). Gyr A, therefore, must be directly involved in DNA synthesis.

Although we are unable to assign a function to Gyr A from our data, it is reasonable to suppose that its function in φX174 stage III replication is related to its known enzymatic activities and its function in *E. coli* replication. Gyr A has the breakage/reunion activity of gyrase (18, 19). It has been proposed to act during the elongation stage of *E. coli* replication by functioning as a "swivelase" (5, 15). This would relieve the positive superhelical stress that accumulates ahead of the replication fork as the DNA strands are separated. It is conceivable that φX174, which utilizes much of the *E. coli* replication machinery, also uses Gyr A as a "swivelase." If Gyr A is acting in such a manner, it must be complexed to another protein because it has no breakage/reunion activity by itself (25, 26). The only other protein besides Gyr B known to interact with Gyr A is a 50,000-dalton protein apparently related to the larger Gyr B protein (25, 26). The topoisomerase formed by this association, called topoisomerase II′, has no supertwisting activity but can relax negative or positive supercoils, making it an attractive candidate for the "swivelase." Its relaxation activity is inhibited by oxolinic acid, a more potent analog of Nal, but not by Novo (25, 26). Although the 50,000-dalton protein may be derived from Gyr B, it is not temperature-sensitive in the ts *gyrB* mutant (M. Gellert, personal communication). Topoisomerase II′ therefore would not be inhibited in this mutant at the nonpermissive temperature but would be inhibited in the *ts gyrA* mutant.

A swivel would seem to be unnecessary for a rolling circle template because any superhelical stress induced by strand separation at the replication fork could be relieved by rotation at the free 3′-OH end. Theoretical calculations suggest that the energy and kinetic barriers to this rotation are small (27–29). Free rotation of the DNA template presumably occurred in the *in vitro* system of Eisenberg *et al.* (8) in which viral strand DNA was synthesized in the absence of Gyr A or any other topoisomerase that could act as a swivel. Besides the presence of more cellular components, our *in vitro* system differs from that of Eisenberg *et al.* in the requirement for the packaging of DNA in order to have stage III DNA synthesis. The association of the phage prohead, or perhaps some other protein, with the replication complex may restrict the free rotation of the DNA and thus introduce the requirement for a swivel. The possibility that a topoisomerase is needed not as a swivel but to catenate the single-stranded DNA as it is being packaged cannot be entirely ruled out but we think this unlikely because there is no evidence that single-stranded DNA isolated from φX174 is ever knotted.

Orr *et al.* (30) have shown previously that a *ts gyrB* mutant is not affected in the elongation stage of *E. coli* replication but is defective in initiation of replication at the nonpermissive temperature, whereas a *ts gyrA* mutant seems to be affected in elongation (15). The result with the *ts gyrB* mutant is in contrast to previous reports of Gyr B inhibitors causing a fast shut-off of DNA replication (20, 31, 32), suggesting a role for Gyr B in elongation. However, in the case of bacteriophage T4 DNA delay mutants, which require Gyr B for DNA synthesis, coumermycin seemed to inhibit initiation, not elongation, of DNA replication (33). Novo also did not seem to affect elongation in an *in vitro* φX174 stage II replication system (34). We can offer no simple explanation for these differing results but the possibility exists that in *E. coli*, Gyr A and Gyr B do not always function in the same complex— i.e., DNA gyrase. Our results indicate that, in φX174, the subunits of DNA gyrase can be utilized separately. Although confirmation of the role of topoisomerase II′ awaits the isolation of a suitable *ts* mutant, we suggest that topoisomerase II′ acts as a swivel during φX174 DNA synthesis.

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