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Decatenation activity of topoisomerase IV during *oriC* and pBR322 DNA replication *in vitro*

(chromosome decatenation/topoisomerase)

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ABSTRACT **Topoisomerase IV (Topo IV), encoded by *parC* and *parE*, is required for partition of the daughter chromosomes in *Escherichia coli*. This enzyme is likely responsible for decatenating the linked daughter chromosomes after replication. In this report, we have examined the action of Topo IV in both pBR322 and *oriC* DNA replication reconstituted *in vitro* with purified proteins. Gyrase fails to decatenate the linked daughter molecules under any condition in the *oriC* system and at physiological salt concentrations in the pBR322 system, whereas Topo IV stimulates generation of monomer product DNA by 7- to 10-fold. Topo IV-catalyzed decatenation of isolated multiply linked DNA dimers was relatively insensitive to salt; it proceeded at 14% of the maximal rate even in the presence of 800 mM potassium glutamate. In contrast, decatenation *in vitro* by gyrase was inhibited completely under these conditions. Pulse–chase analysis indicated that Topo IV-catalyzed resolution of linked daughter DNA molecules occurred prior to completion of DNA replication, such that multiply linked daughter molecules did not arise. These results suggest that during DNA replication, gyrase acts primarily to relieve accumulated positive supercoiling and Topo IV acts to segregate the daughter chromosomes.**

Replication of circular chromosomes leaves the daughter chromosomes linked topologically in the form of catenanes (1). In bacteria, successful cell division requires that the daughter chromosomes be disengaged topologically (decatenation), presumably by the action of a topoisomerase, and that the daughter nucleoids be separated topographically (partition), one migrating to each of the daughter cells (2).

Six mutations (*parA–F*) have been identified that show a defect in these processes (2). *parA* and *parD* were allelic to *gyrB* and *gyrA*, respectively (3–5), whereas *parB* was allelic to *dnaG* (6). The identification of the genes encoding the two subunits of DNA gyrase as *par* loci was consonant with the observation that in mutant strains that were temperature-sensitive in *gyrB*, the nucleoid appeared dumbbell-shaped and twice the normal size (7), suggesting that topological resolution of the daughter chromosomes had not occurred. Schmid (8) and Kato *et al.* (9) demonstrated that *parE* was linked closely to *parC*, and Kato *et al.* (10) demonstrated that both purified *Escherichia coli* ParC and ParE were required to reconstitute topoisomerase IV (Topo IV) activity. *parC* and *parE* showed considerable homology to *gyrA* and *gyrB*, respectively (9, 11). A sixth *par* locus, *parF*, could be found just downstream of *parC* (8). Luttinger *et al.* (11) suggested that ParF, which has a hydrophobic amino-terminal region, might serve to anchor Topo IV to the membrane.

The role of Topo IV in decatenating replication intermediates has been supported by the studies of Adams *et al.* (12) that showed that replication catenanes of pBR322 plasmid DNA accumulated at the nonpermissive temperature only in *parC* and *parE* strains, not in *gyrA*, *gyrB*, or *parF* strains.

In this report, we have examined the action of Topo IV in plasmid replication systems reconstituted *in vitro* with purified proteins. We show that Topo IV is required for the topological resolution of the daughter DNA molecules in both unidirectionally and bidirectionally replicating systems, that this decatenation step occurs prior to the completion of DNA replication, and that Topo IV-catalyzed decatenation of multiply linked DNA dimers is less sensitive to salt than that catalyzed by DNA gyrase.

MATERIALS AND METHODS

Replication Proteins and DNAs. ParC and ParE were purified as will be described elsewhere (25). Topo IV was reconstituted by mixing equimolar amounts of ParC and ParE and incubating at 0°C for 30 min. The preparation of DnaA and HU was described by Parada and Marians (13); all other replication proteins were as described by Minden and Marians (14) and Wu *et al.* (15). Supercoiled pBROTB plasmid, harbored in *E. coli* K38*tus* was prepared according to Marians *et al.* (16). The construction of pBROTB will be described elsewhere (H. Hiasa and K.J.M.).

DNA Replication. A standard *oriC* replication reaction mixture (12.5 μl) contained 40 mM Hepes–KOH (pH 8.0), 10 mM $Mg(OAc)_2$, 10 mM dithiothreitol, bovine serum albumin at 100 μg/ml, 2 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 40 μM [α-^{32}P]dATP (3000–10,000 cpm/pmol), 40 μM dGTP, 40 μM dCTP, 40 μM TTP, 4 mM phosphocreatine, 250 ng of creatine kinase, 50 μM NAD, DNA template (35 fmol), 200 ng of DnaA, 150 ng of DnaB, 60 ng of DnaC, 160 ng of DnaG, 1.0 unit of DNA polymerase III*, 25 ng of DnaN, 250 ng of SSB, 10 ng of HU, and 50 ng of DNA gyrase. Where indicated, 0.2 ng of RNase H, 13 ng of DNA ligase, and 24 ng of DNA polymerase I were included. Reaction mixtures were assembled on ice, and the reaction was started by adding DnaA. Incubation was at 30°C for 15 min. A standard pBR322 replication reaction mixture (12.5 μl) contained the same nucleotide and buffer components as the *oriC* reaction mixtures and 0.24 μg of RNA polymerase, 24 ng of DNA polymerase I, 0.2 ng of RNase H, 125 ng of SSB, 110 ng of DnaB, 28 ng of DnaC, 40 ng of DnaG, 14 ng of DnaT, 15 ng of PriA, 4 ng of PriB, 4 ng of PriC, 1.0 unit of DNA polymerase III*, 25 ng of DnaN, 50 ng of DNA gyrase, and DNA template (35 fmol). Reaction mixtures were assembled on ice, and the reaction was started by adding RNA polymerase. Incubation was at 30°C for 20 min. Replication reactions were terminated by the addition of EDTA to 25 mM. A portion of the reaction mixtures was precipitated with trichloroacetic acid and filtered through glass fiber filters, and total DNA synthesis was measured by liquid scintillation

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Abbreviations: Topo III and IV, topoisomerases III and IV; LRI, late replicative intermediate; SC Di, supercoiled DNA dimers.

spectrometry. Another portion of the reaction mixtures was mixed with one-fifth volume of a dye mixture containing 50 mM EDTA, 12.5% glycerol, 2% sarkosyl, 0.05% xylene cyanol, and 0.05% bromophenol blue and analyzed by electrophoresis through vertical 0.8% (or 1.0%) agarose (SeaKem ME; FMC) gels (14 × 10 × 0.3 cm) at 2 V/cm for 16 h in an electrophoresis buffer of 50 mM Tris·HCl (pH 7.9 at 23°C), 40 mM NaOAc, and 1 mM EDTA. Gels were dried under vacuum onto Whatman 3M paper and autoradiographed with Amersham Hyperfilm-MP.

Decatenation of Multiply Linked DNA Dimers. Multiply linked DNA dimers were purified as described by Marians (17) by sucrose gradient centrifugation from *oriC* replication reactions (in the absence of Topo IV) increased in size by 120-fold. Decatenation reaction mixtures (5 μl) containing 50 mM Hepes–KOH (pH 8.0), 6 mM $MgCl_2$, 5 mM dithiothreitol, bovine serum albumin at 100 μg/ml, DNA dimers (4 fmol as monomer), and 40 fmol of Topo IV were incubated at 30°C for 2.5 min. The reactions were stopped by the addition of EDTA to 20 mM. Gel loading dye was then added, and the reactions were analyzed by electrophoresis through vertical 0.8% agarose gels at 2 V/cm for 18 h. The gels were dried and autoradiographed.

RESULTS

Topo IV-Catalyzed Segregation of Replicating Daughter DNA Molecules. The template used for these studies was pBROTB, a 6-kb pBR322 plasmid that carries *oriC* [including the A+T-rich region to the left of the 13-mers (18)] and two *TerB* sequences (19) with a 1-kb spacer between them. Thus, pBROTB can be replicated via either a pBR322-type or *oriC*-type initiation mechanism, depending on the replication proteins provided.

Topoisomerase function is required, during the replication of a circular DNA, for removal of the positive supercoils generated by the advancing replication fork and for the topological resolution of the linked daughter molecules. Prior to the discovery of Topo IV (9, 10), of the *E. coli* topoisomerases, only gyrase was found to be capable of removing positive supercoils (by converting them directly to negative ones) (20). Because Topo IV is capable of relaxing positive supercoils (10), we asked whether it could support replication fork movement in a plasmid DNA. We found that Topo IV was unable to support incorporation of labeled precursor into acid-insoluble product with either the pBR322 or *oriC* DNA replication system (data not shown). We therefore focused on the question of whether Topo IV could catalyze the segregation of replicating daughter DNA molecules.

Gyrase is incapable of catalyzing the decatenation of multiply linked DNA dimers in the presence of physiological concentrations of salt (17). Surprisingly, gyrase generated little monomer product during *oriC* replication reconstituted with the pBROTB template in the presence of DnaA, DnaB, DnaC, DnaG, HU, SSB, and the DNA polymerase III holoenzyme, even though there was no exogenous salt present (Fig. 1). The bulk of the product appeared as form II:form II DNA dimers in the absence of DNA ligase (Fig. 1*A*, lane 1) and supercoiled form I:form I DNA dimers (SC Di) in the presence of DNA ligase (Fig. 1*A*, lane 6). Late replicative intermediate (LRI) was also evident in each case. On the other hand, Topo IV stimulated the accumulation of monomer product by 7- to 9-fold without affecting the overall level of DNA replication (Fig. 1). Maximal stimulation occurred at a ratio of Topo IV to template of 2.8:1. The final product in the presence of DNA ligase was form I DNA (Fig. 1*A*, lanes 7–10) and form II DNA in the absence of DNA ligase (Fig. 1*A*, lanes 2–5).

Plasmids carrying *oriC* replicate bidirectionally. To determine if Topo IV could also segregate daughter molecules generated in a unidirectionally replicating system, we examined its action during pBR322 DNA replication. In the absence of added salt, gyrase is capable of completely decatenating pBR322 replication intermediates; however, it fails to do so in the presence of physiological concentrations of salt (refs. 14 and 21; H. Hiasa, R. J. DiGate, and K.J.M., unpublished results). pBR322 DNA replication was reconstituted in the presence of 200 mM potassium glutamate with RNA polymerase, RNase H, DNA polymerase I, SSB, DnaB, DnaC, DnaG, DnaT, PriA, PriB, PriC, and the DNA polymerase III holoenzyme (Fig. 2). In the presence of only gyrase, little form II DNA accumulated; the bulk of the product was present as form II:form II DNA dimers. As in the case of *oriC* replication, the addition of Topo IV stimulated the accumulation of monomer form II product by 10-fold, whereas the overall level of DNA replication was reduced by about 40% (Fig. 2). Thus, Topo IV is capable of catalyzing the segregation of replicating daughter DNA molecules under a variety of conditions.

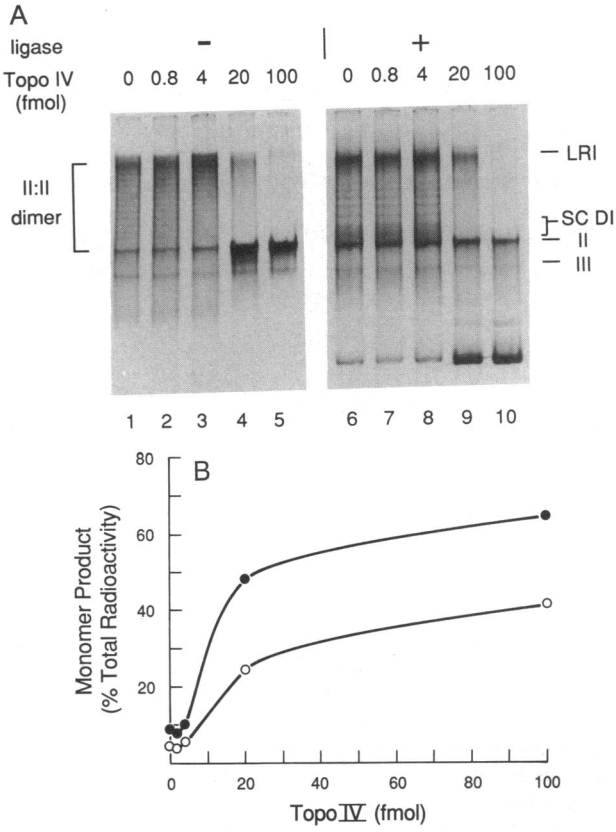

FIG. 1. Topo IV-catalyzed decatenation of replicating daughter DNA molecules during *oriC* DNA replication. (*A*) Standard *oriC* DNA replication reaction mixtures containing RNase H, DNA polymerase I, and the indicated amounts of Topo IV were incubated in the presence (lanes 6–10) or absence (lanes 1–5) of DNA ligase, processed, and analyzed as described in *Materials and Methods*. Shown is an autoradiogram of the DNA products analyzed by electrophoresis through a 0.8% agarose gel. (*B*) The autoradiogram of the gel was scanned with a Millipore Bioimage densitometer, and the fraction of the total activity present as monomer product (form II and form I DNA in the absence and presence of DNA ligase, respectively) was determined. ○, replication reactions in the absence of DNA ligase (lanes 1–5 in *A*); ●, replication reactions in the presence of DNA ligase (lanes 6–9 in *A*). There were 37.7 pmol, 34.9 pmol, 33.6 pmol, 34.3 pmol, 26.6 pmol, 50.9 pmol, 46.7 pmol, 51.5 pmol, 51.7 pmol, and 37 pmol of [α-^{32}P]dATP incorporated into acid-insoluble product in the reactions shown in lanes 1–10, respectively. II:II dimer, form II:form II DNA dimers; SC Di, form I:form I DNA dimers; I, II, and III, form I, form II, and form III DNA, respectively.

Topo IV-Catalyzed Decatenation of Multiply Linked DNA Dimers Proceeds at High Salt Concentrations. As mentioned

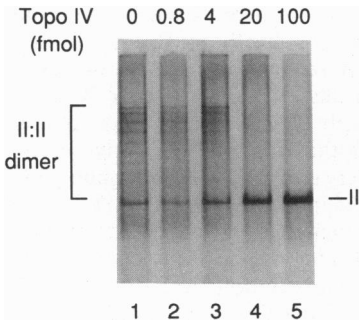

FIG. 2. Topo IV-catalyzed decatenation of replicating daughter DNA molecules during pBR322 DNA replication. Standard pBR322 DNA replication reaction mixtures containing the indicated amounts of Topo IV were incubated, processed, and analyzed as described in *Materials and Methods*. Shown is an autoradiogram of the DNA products analyzed by electrophoresis through a 0.8% gel. Five and nine-tenths percent and 61.1% of the total radioactivity were present as monomer product in lanes 1 and 5, respectively. There were 11.2 pmol, 6.6 pmol, 7.6 pmol, 7.3 pmol, and 6.1 pmol of [α-^{32}P]dATP incorporated into acid-insoluble product in the reactions shown in lanes 1–5, respectively.

above, the failure of gyrase to successfully resolve replicating pBR322 DNA daughter molecules (14, 21) was attributed to the salt sensitivity of gyrase-catalyzed decatenation of multiply linked DNA dimers (17). If Topo IV is the primary decatenase in the cell, it must be capable of catalyzing this reaction at concentrations of potassium glutamate that normally range between 150 and 400 mM and can be as high as 800 mM (22). This was tested directly.

Multiply linked DNA dimers with an average intermolecular linking number (17) of 16–17 were prepared as described in *Materials and Methods*. The rate of Topo IV-catalyzed decatenation was maximal at a molar ratio of Topo IV to DNA monomers of 10 (data not shown). The effect of increasing concentrations of potassium glutamate on the decatenation rate was then examined (Fig. 3). The rate of decatenation was determined as described by Marians (17). The rate of Topo IV-catalyzed decatenation of multiply linked DNA dimers remained maximal up to 200 mM potassium glutamate and then started to decrease (Fig. 3). However, even at 800 mM potassium glutamate, the decatenation rate was substantial; it was 14% of the maximal value. This indicates that Topo IV should be competent to decatenate daughter chromosomes in the cell under essentially all osmotic conditions that might be encountered.

Topo IV-Catalyzed Decatenation of Daughter DNA Molecules Occurs Prior to the Completion of DNA Replication. There are two basic pathways that can be followed during topoisomerase-catalyzed segregation of replicating intermediates (1, 21). Either the linkages generated between the daughter molecules are removed as the replication fork traverses the final region of parental DNA in the late, Cairn's-type replication intermediate, or they are removed after replication is completed. In the latter case, the segregation pathway is therefore LRI → multiply linked supercoiled DNA dimers (SC Di) → form I DNA, whereas in the former case, no SC Di would be observed. During pBR322 DNA replication in the absence of salt, gyrase-catalyzed segregation follows the former pathway (21). We used pulse–chase analysis to determine the pathway favored when Topo IV was present during *oriC* DNA replication (Fig. 4).

In the absence of Topo IV, DNA product accumulated as SC Di (Fig. 4*A*). There was some final form I product generated, amounting to 0.7%, 4.4%, and 6.1% of the total radioactivity after 4, 8, and 16 min, respectively, of the chase (Fig. 4*A*, lanes 4, 5, and 6, respectively). At the lowest level of Topo IV examined (2.5 fmol, Fig. 4*B*), the SC Di persisted,

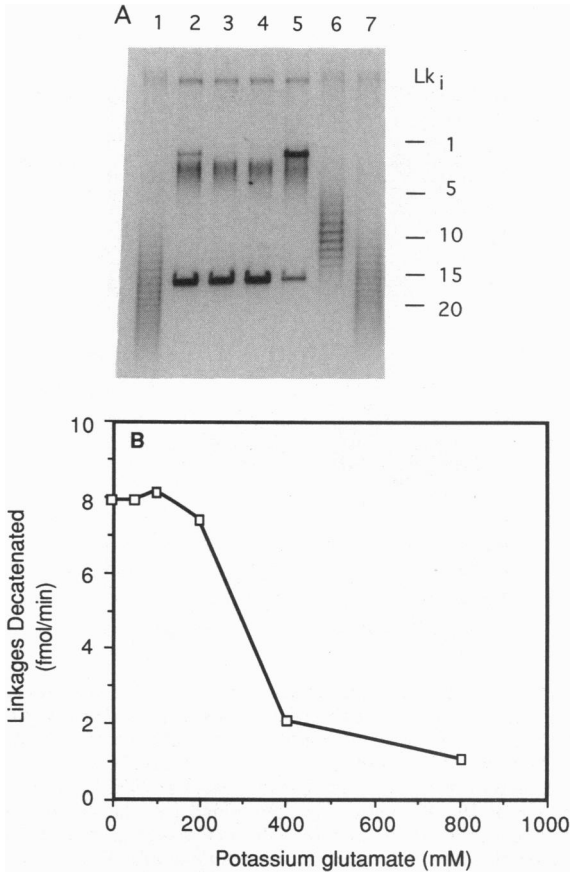

FIG. 3. Effect of potassium glutamate on Topo IV-catalyzed decatenation of multiply linked, form II:form II DNA dimers. (*A*) Standard decatenation reaction mixtures containing either no Topo IV (lane 1) or 40 fmol of Topo IV (lanes 2–7) and either no (lanes 1 and 2) or 50 mM, 100 mM, 200 mM, 400 mM, or 800 mM (lanes 3–7) potassium glutamate were incubated, processed, and analyzed as described in *Materials and Methods*. Shown is an autoradiogram of the analysis by electrophoresis through a 0.8% agarose gel. (*B*) Quantitation of the decatenation reaction. The autoradiogram was scanned as in the legend of Fig. 1, and the amount of linkages present in each lane was calculated as described by Marians (17). The decatenation rates were then derived from this data. Lk_i, intermolecular linking number.

although significant amounts of form I product were observed after only 2 min of the chase. At higher levels of Topo IV (5 fmol, Fig. 4*C*; and 10 fmol, Fig. 4*D*), no SC Di could be observed at any time during the chase. Formation of monomer product was stimulated 60-fold by 10 fmol of Topo IV after 4 min of the chase (compare Fig. 4*A*, lane 4 to Fig. 4*D*, lane 4, where 42.7% of the total radioactivity was present as monomer product). The absence of significant accumulation of SC Di in the presence of low levels of Topo IV suggests that either Topo IV resolves the linkages between the daughter DNA molecules as they arise and prior to the completion of DNA replication or SC Di arise but are decatenated too quickly to be observed during the chase. The former explanation seems much more likely because the level of Topo IV (5 fmol) at which SC Di can no longer be observed during the chase (Fig. 4*C*) is far below the levels required to saturate either decatenation of the daughter molecules during replication (Fig. 1*B*) or decatenation of purified multiply linked DNA dimers (Fig. 3*B*).

DISCUSSION

The action of Topo IV in *oriC* and pBR322 plasmid DNA replication systems *in vitro* has been examined. Even though

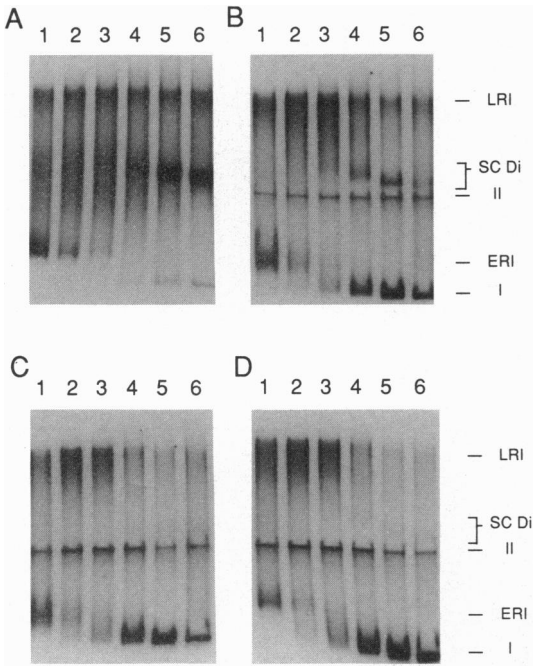

FIG. 4. Pulse–chase analysis of Topo IV-catalyzed segregation of replicating *oriC* DNA daughter molecules. Standard *oriC* replication reaction mixtures were increased in size 4-fold and contained either no Topo IV (*A*) or 2.5 fmol (*B*), 5 fmol (*C*), or 10 fmol (*D*) of Topo IV. Reaction mixtures were first incubated at 30°C for 2 min in the absence of DnaA. DnaA was then added and the incubation was continued for another 2 min. [α-^{32}P]dATP was then added (to 10,000 cpm/pmol), and the incubation was continued for 1 min. Unlabeled dATP was then added to a final concentration of 4 mM. Aliquots (6.25 μl) were withdrawn immediately after the addition of unlabeled DNA (0 min, lane 1) and subsequently after 1, 2, 4, 8, and 16 min (lanes 2–6, respectively), and the reaction was terminated by mixing with an equal volume of 50 mM EDTA. Loading dye was then added, and the reactions were analyzed by electrophoresis through 0.8% agarose gels in the presence of ethidium bromide at 25 ng/ml. On average, 2 pmol of [α-^{32}P]dATP was incorporated into acid-insoluble material during the pulse. Acid-insoluble cpm remained constant in all cases during the chase. ERI, early replication intermediate.

Topo IV is capable of relaxing positive supercoils (10), it was unable to support DNA replication in the absence of DNA gyrase. On the other hand, Topo IV proved very effective in catalyzing the resolution of linked daughter DNA molecules.

In our initial studies on pBR322 DNA replication *in vitro*, we noted that DNA gyrase failed to segregate the replicating daughter DNA molecules (14). We showed subsequently (17), using multiply linked DNA dimers purified from replication reactions as substrate for gyrase-catalyzed decatenation reactions, that whereas gyrase could catalyze this reaction, decatenation was inhibited by levels of salt [80 mM KCl (17) or 150 mM potassium glutamate (H. Hiasa, R. J. DiGate, and K.J.M., unpublished results)] that approximated intracellular concentrations. This explained the original observation but called into question the paradigm that gyrase was responsible for decatenating the daughter chromosomes in the cell (23).

Additional studies from this laboratory established that Topo III was perfectly capable of segregating the daughter molecules *in vitro*, even at 800 mM potassium glutamate (ref. 24; H. Hiasa, R. J. DiGate, and K.J.M., unpublished results). The problem here, of course, was that *E. coli* strains carrying null mutations of *topB* (encoding Topo III) were viable, suggesting that its role, if any, during chromosomal DNA replication was subtle.

The answer to the question of which topoisomerase in the cell segregates the daughter chromosomes appears to be Topo IV. As shown here, this enzyme stimulated monomer product formation by 7- to 10-fold during both *oriC* and pBR322 DNA replication *in vitro* and as much as 60-fold during pulse–chase analysis. Topo IV-catalyzed decatenation of multiply linked dimers was significantly less salt-sensitive than that of gyrase; it decreased by only a factor of 7 in the presence of 800 mM potassium glutamate. Thus, as befits the cellular decatenase, Topo IV is capable of resolving replicating daughter DNA molecules at the highest concentration of salt likely to be experienced *in vivo* (22).

Nevertheless, mutations in *gyrA* and *gyrB* do show a *par* phenotype (3–5). If this is not a result of the direct participation of gyrase in chromosome decatenation, how might this phenotype be explained? One possibility is that the phenotype arises as a result of the cessation of replication fork movement caused by inactivating the gyrase. This might act to uncouple coordination between the replication and partition machinery, thereby generating the *par* phenotype.

Pulse–chase analysis showed that Topo IV-catalyzed decatenation of the linked daughter molecules occurred in concert with movement of the replication fork through the final region of nonreplicated parental DNA in the LRI. Thus, Topo IV acted on the linkages as they arose. This is unlike the case for gyrase, which, during pBR322 DNA replication in the absence of salt, acts on the accumulated multiply linked dimers, but is similar to the case for Topo III action during both pBR322 and *oriC* DNA replication (H. Hiasa, R. J. DiGate, and K.J.M., unpublished results). Perhaps this suggests that both Topo III and Topo IV interact with a component of the replication fork, whereas DNA gyrase cannot do so.

Surprisingly, even in the absence of intracellular levels of salt, gyrase was incapable of generating significant levels of monomer product in the *oriC* replication system, whereas it could do so, under these conditions, in the pBR322 replication system. It is interesting to speculate that the reason for this may be that replication in the former is bidirectional, whereas in the latter, it is unidirectional. Perhaps in the bidirectional system, the linkages between the daughter molecules are confined to the region between the two advancing replication forks, creating a poor substrate for DNA gyrase, but one that served Topo IV well. In a unidirectional system, the linkages would not be inhibited from diffusing freely across the daughter molecules and would thus be accessible for either topoisomerase.

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