Roles of DNA Topoisomerases in the Regulation of R-loop Formation in Vitro*

(Received for publication, August 22, 1996, and in revised form, November 2, 1996)

Pauline Phoenix, Marc-André Raymond, Éric Massé, and Marc Drolet†
From the Département de Microbiologie et immunologie, Université de Montréal, C.P. 6128, Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada

We have recently found that stable R-loop formation occurs in vivo and in vitro when a portion of the Escherichia coli rnrB operon is transcribed preferentially in its physiological orientation. Our results also suggested that the formation of such structures was more frequent in topA mutants and was sensitive to the template DNA supercoiling level. In the present report we investigated in greater detail the involvement of DNA topoisomerases in this process. By using an in vitro transcription system with phage RNA polymerases, we found that hypernegative supercoiling of plasmid DNAs in the presence of DNA gyrase is totally abolished by RNase H, suggesting that extensive R-looping occurs during transcription in the presence of DNA gyrase. When RNase A is present, significant hypernegative supercoiling occurs only when the 567-base pair rnrB HindIII fragment is transcribed in its physiological orientation. This result suggests that more stable R-loops are being produced in this orientation. Our results also suggest that DNA gyrase can participate in the process of R-loop elongation. The strong transcription-induced relaxing activity of E. coli DNA topoisomerase I is shown to efficiently counteract the effect of DNA gyrase and thus inhibit extensive R-looping. In addition, we found that an R-looped plasmid DNA is a better substrate for relaxation by E. coli DNA topoisomerase I as compared with a non-R-looped substrate.

DNA topoisomerases are enzymes involved in the regulation of DNA conformation (for a review see Ref. 1). Since the topology of DNA affects most of its functions, DNA topoisomerases are considered key elements in the regulation of genomic activities. In bacteria, they are involved in the control of chromosomal DNA supercoiling (2). This task is apparently performed by two DNA topoisomerases with opposite enzymatic activities: DNA topoisomerase I (originally known as ω protein), encoded by the topA gene that specifically relaxes negative supercoiling (3), and DNA gyrase encoded by the gyrA and gyrB genes that introduces negative supercoiling into the DNA (4). The involvement of DNA topoisomerases in every step of DNA replication, namely initiation, elongation, and termination is also well known in bacteria (5). Although much is known about the role of DNA topoisomerases in transcription initiation through the modulation of DNA supercoiling level (6), little is known about their involvement in the two following steps, namely elongation and termination.

The tight association of DNA topoisomerases with actively transcribed genes has been well-described in eukaryotes (7–13). In bacteria, changes in plasmid DNA supercoiling in topoisomerase mutants have been linked to transcription elongation in some cases (14, 15). Hence, the production of positively supercoiled plasmid DNA upon DNA gyrase inactivation (16) is linked to transcription elongation (15). In the absence of the topA gene function, the production of hypernegatively supercoiled plasmid DNA (17) has also been linked to transcription elongation (14). However, only transcription of genes encoding for membrane-bound proteins are shown to generate such hypernegative supercoiling (18). One exception is the galK gene which, upon transcription in cis, can be responsible for hypernegative supercoiling of plasmid DNAs (19). In order to explain the link between DNA topoisomerases and transcription elongation in both eucaryotes and procaryotes, a twin-domain model for transcription has been proposed (20). According to this model, positive and negative supercoiling domains are generated, respectively, ahead and behind the moving RNA polymerase. This model has been supported by much experimental evidence (for a review see Ref. 21). Predicted by this model is the fact that co-transcription and translation with simultaneous attachment of the nascent peptide to the membrane will generate the maximum number of supercoils, because in this situation the rotation of the elongation complex is completely blocked. This model also explains why positive supercoiling will be generated during transcription in the absence of DNA gyrase, since, in this situation, only the negative supercoils will be removed by DNA topoisomerase I. Hypernegative supercoiling in the absence of DNA topoisomerase I is equally explained by the selective removal of positive supercoils by DNA gyrase.

Interestingly, the R-loop structure possesses all the elements to...
be an excellent substrate for DNA topoisomerase I activity, namely a single-stranded DNA region in proximity of a double-stranded one (24).

In order to better study the putative roles of DNA topoisomerases in the regulation of R-loop formation, it was necessary to isolate DNA sequences that are subject to forming such structures upon transcription. We recently showed that stable R-loop formation occurs during transcription of a portion of the rrnB operon, preferentially in its physiological orientation. This study also showed that DNA supercoiling was an important factor for R-loop formation. In the present report, we investigated in greater detail the involvement of DNA topoisomerases and DNA supercoiling in R-loop formation during transcription of this rrnB DNA fragment.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pJP459 and pJP461 have been described. Briefly, they are pBluescript II KS derivatives in which the 567-base pair HindIII fragment from the rrnB operon of E. coli was inserted in the reverse (pJP459) or the physiological (pJP461) orientation relative to the vector map numbering. For in vitro transcription experiments, these plasmids were purified by using the Qiagen plasmid kit (Qiagen).

**Enzymes and Chemicals**—T3 and T7 RNA polymerases were from Stratagene. E. coli DNA gyrase A and B subunits and E. coli DNA topoisomerase I were generously provided by James C. Wang (Harvard University) and Leroy F. Liu (University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School). DNA gyrase was reconstituted by preincubating at 37 °C for 30 min. Gyra (1.42 μg) and GyrB (1.5 μg) subunits together in a total volume of 30 μl of a solution containing 50 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 5 mM dithiothreitol, 70 mM KCl, and 50% glycerol. 1 μl of this mixture (i.e., about 50 ng each of Gyra and GyrB) was used in a typical in vitro transcription reaction (see below). E. coli RNase H1 was kindly provided by R. J. Crouch (National Institutes of Health). RNase A, from Boehringer Mannheim, was prepared as described (25). Proteinase K was from Boehringer Mannheim.

**In Vitro Transcription Reactions**—Typical in vitro transcription reactions were performed essentially as described previously (22). Briefly, they were performed in a volume of 25 μl of a solution containing 35 mM Tris (pH 8.0), 25 mM MgCl2, 20 mM KCl, 0.4 mM each of CTP, GTP, and UTP, 1.2 mM ATP, 0.5 μg of purified pJP459 or pJP461 DNA, 10 units of T3 or T7 RNA polymerase, and when specified, RNase A and RNase H were added at the indicated concentrations. Unless otherwise indicated, the reactions were incubated at 37 °C for 3 min before the addition of E. coli RNase H (about 50 ng of each subunit) and then incubated for an additional 10 min at the same temperature. Reactions were terminated by the addition of 25 μl of a solution containing 50 mM EDTA, 1% SDS, and 12.5 μg of proteinase K. After an incubation of 30 min at 37 °C, the samples were extracted once with phenol and once with chloroform and then precipitated with ethanol. They were resuspended in 20 μl of a reaction buffer containing 10 mM Tris (pH 8.0), 10 mM MgCl2, 100 mM NaCl, 1 μg of RNase A, and 20 ng of RNase H. After an incubation of 45 min at 37 °C, the samples were phenol-extracted and then analyzed by one-dimensional agarose gel electrophoresis (0.8%) with chloroquine (15 μM) in 0.5 × TBE (25). After electrophoresis, they were either soaked in 1 mM MgCl2 for 1 h before being stained with ethidium bromide or they were dried and prepared for in situ hybridization as described (15). Two-dimensional agarose gel electrophoresis was performed as described previously (22) except that 0.5 × TBE was used instead of TPE during electrophoresis. The chloroquine concentrations used were 15 and 60 μM, respectively, in the first and second dimension. After electrophoresis, gels were dried and prepared for in situ hybridization as described (15).

**RESULTS**

The Formation of Hypernegatively Supercoiled Plasmid DNAs during Transcription by a Phage RNA Polymerase in the Presence of DNA Gyrase Involves R-loop(s)—R-loop formation was recently shown to occur preferentially when the 567-base pair HindIII fragment from the rrnB operon of E. coli was transcribed in its physiological orientation in a topA mutant and in an in vitro system using phage RNA polymerases. This conclusion is based on the fact that RNase H-sensitive gel retardation of plasmid DNAs during electrophoresis was observed when this fragment was preferentially transcribed in its original orientation, either in vitro or in vivo. R-loop formation during transcription of this DNA fragment in the topA mutant was also shown to be sensitive to the DNA supercoiling level. Previous in vitro studies have shown that hypernegatively supercoiling of pBR322 DNA in the presence of DNA gyrase and during transcription by E. coli RNA polymerase was linked to R-loop formation (22). We thus considered the possibility that the hypernegative supercoiling assay could be used to demonstrate the orientation preference in the process of R-loop formation during transcription of the rrnB HindIII fragment (see below). In addition, in order to study the role of DNA gyrase in R-loop formation during transcription of the HindIII fragment from the rrnB operon, it was important to find experimental conditions allowing both active transcription by phage RNA polymerases and DNA supercoiling by DNA gyrase. In the beginning we used the pJP459 plasmid, a pBluescript II KS derivative in which the rrnB HindIII fragment is oriented in such a way that it is transcribed in its physiological orientation by the T3 RNA polymerase. As can be seen in Fig. 1, transcription by T3 RNA polymerase allows the production of hypernegatively supercoiled pJP459 DNA in the presence of DNA gyrase (complete). The omission of one nucleotide completely abolished hypernegative supercoiling (3 NTPs), showing that active transcription is required. Hypernegative supercoiling of pJP459 DNA seems to be totally dependent on R-loop formation, since it is completely abolished by the presence of RNase H during transcription (complete + RNase H). The RNase H effect is better seen in the bottom panel, where the hypernegatively supercoiled topoisomers revealed by two-dimensional agarose gel electrophoresis (complete) are totally absent when RNase H is added during transcription (RNase H). An aliquot of the DNA sample loaded on the two-dimensional gel (complete) was shown to be completely relaxed by E. coli DNA topoisomerase I demonstrating that it is indeed hypernegatively supercoiled DNA (data not shown). In a separate experiment, by using relaxed pJP459 DNA, we have shown that the DNA supercoiling activity of DNA gyrase is not affected at all by the presence of RNase H under the transcription conditions used (data not shown). In addition, RNase H had no inhibitory effect on transcription, and indeed, as previously demonstrated, the production of RNA increased in the presence of RNase H, suggesting that an R-loop can act as a roadblock for a transcription complex (data not shown). Interestingly, the presence of RNase A at high concentration had no effect on the formation of hypernegatively supercoiled pJP459 DNA (complete + RNase A).

Hypernegative Supercoiling in the Presence of RNase A Occurs Preferentially when the rrnB HindIII Fragment Is Transcribed in Its Physiological Orientation—We considered the possibility that the hypernegative supercoiling assay might have some advantages over the gel shift assay to demonstrate R-loop formation. Indeed, in the hypernegative supercoiling assay, an RNase H-dependent linking number change that occurs before the arrest of transcription is taken as evidence for R-loop formation, and it is thus independent of the stability of the R-loop during electrophoresis. In order to demonstrate that hypernegative supercoiling occurs preferentially when the rrnB HindIII fragment is transcribed in its physiological orientation, time course experiments were performed. In the beginning, on account of the observation that the production of hypernegatively supercoiled plasmid DNA was not as fast when T7 RNA polymerase was used instead of T3 RNA polymerase, we decided to use the T7 RNA polymerase to facilitate
Regulation of R-loop Formation by DNA Topoisomerases

Hypernegative supercoiling of plasmid DNAs during transcription by T3 RNA polymerase in the presence of DNA gyrase involves R-loop(s). Transcription reactions using T3 RNA polymerase and pJP459 DNA were performed as described under “Experimental Procedures,” and DNA samples in the top and bottom panels were, respectively, analyzed by one-dimensional and two-dimensional agarose gel electrophoresis. Top panel, the first lane shows the complete reaction in which transcription was allowed to proceed at 37 °C for 3 min before the addition of DNA gyrase and another incubation of 10 min. The second lane is as the first lane except that RNase H (20 ng) was added in the beginning of the reaction. The third lane is as the first lane except that UTP was omitted in the reaction. The fourth lane is as the third lane except that RNase H (20 ng) was added in the beginning of the reaction. The fifth lane is as the first lane except that T3 RNA polymerase was omitted in the reaction. The sixth lane is as the fifth lane except that RNase H (20 ng) was added in the beginning of the reaction. The seventh lane is as the first lane except that RNase A (40 μg/ml) was added in the beginning of the reaction. The eighth lane is as the first lane except that both RNase H (20 ng) and RNase A (40 μg/ml) were added in the beginning of the reaction. The ninth lane is as the first lane except that DNA gyrase was not added. After electrophoresis, the gel was stained with ethidium bromide. [ – ] indicates the position of hypernegatively supercoiled DNAs in the gel. Bottom panel, complete and RNase H show, respectively, the same as the first and second lanes of the top panel. Under the chloroquine concentrations used, hypernegatively supercoiled plasmid DNAs migrate at the end of the left part of the curve.

The analysis of the results, pJP459 and pJP461 DNAs were thus transcribed by T7 RNA polymerase in the presence of DNA gyrase, and aliquots were periodically taken and mixed with a stop solution as described under "Experimental Procedures." In pJP459, T7 RNA polymerase transcribes the rnrB HindIII fragment in its reverse orientation, whereas in pJP461, this DNA fragment is transcribed in its physiological orientation by the T7 RNA polymerase. As can be seen in Fig. 2A, the appearance of hypernegatively supercoiled topoisomers increased with time and was relatively rapid, since within 2 min a significant amount of topoisomers were already hypernegatively supercoiled. No differences were observed between pJP459 and pJP461 DNAs, suggesting that hypernegatively supercoiling, and hence R-looping, was rather nonspecific under these conditions. However, when RNase A at 20 μg/ml was added during the time course experiment (Fig. 2B), almost no hypernegatively supercoiled topoisomers were produced when pJP459 was transcribed (left part), whereas such topoisomers were clearly produced when the rnrB HindIII fragment was transcribed in its physiological orientation (pJP461; right part). These results suggest that the process of R-loop formation during transcription of the rnrB HindIII fragment in its physiological orientation is very resistant to RNase A at a high concentration and therefore that the R-loops are more stable and/or that they are generated at a higher frequency. We consider unlikely the possibility that the RNase A effect at this high concentration is linked to degradation of nascent RNAs that can theoretically participate in R-loop formation in a 3’ to 5’ direction, because if this were the case, then R-loop formation and hence hypernegatively supercoiling should always be sensitive to RNase A at this concentration, irrespective of the transcribed DNA template. Therefore, we believe that R-loop formation during transcription by phage RNA polymerases occurs in a 5’ to 3’ direction and that the RNA in the hybrid is elongated by RNA polymerase without being displaced from the template strand. This is also suggested from experiments described below.

Highly Negatively Supercoiled Plasmid DNAs Are Produced in the Presence of DNA Gyrase after the Arrest of Transcription—In order to investigate in greater detail the role of DNA gyrase in R-loop formation, we designed a two-stage reaction in which DNA gyrase is added after transcription is arrested. A similar approach has been used to demonstrate R-loop formation due to transcription of pBR322 DNA in the presence of DNA gyrase (22). pJP459 and pJP461 DNAs were thus transcribed with T3 or T7 RNA polymerase, and after the arrest of transcription by heating, DNA gyrase was added. The results of such experiments are presented in Fig. 3. Under the chloroquine concentration used during electrophoresis (7.5 μg/ml; Fig. 3A), an irregular distribution of topoisomers (e.g. second lane) reflects a population where more negatively supercoiled topoisomers are present as compared with a regular distribution of topoisomers (e.g. first lane), whereas less negatively supercoiled topoisomers are present. This was confirmed by two-dimensional agarose gel electrophoresis (Fig. 3, B and C). As can be seen in Fig. 3A, in the absence of any added RNases, similar topoisomers distributions are observed, irrespective of the gyrase and the plasmid DNA used. These distributions are highly heterogeneous, with some topoisomers being highly negatively supercoiled. This is better seen in Fig. 3B (pJP459, no RNases) and Fig. 3C (pJP461, no RNases), where some DNA samples were loaded on a two-dimensional agarose gel. The fact that RNase H completely eliminated this heterogeneity and the formation of highly negatively supercoiled topoisomers shows that this is due to R-loop formation (Fig. 3, A–C, +RNase H). Interestingly, when RNase A was added, more negatively supercoiled topoisomers were produced with T3 RNA polymerase than with T7 RNA polymerase in the case of pJP459 (Fig. 3, A and B, +RNase A), whereas the reverse situation was observed for pJP461 (Fig. 3, A and C, +RNase A). This is expected if transcription of the rnrB HindIII fragment in its physiological orientation produces R-loops that are more stable. We consider unlikely that this is an indication that R-loop formation is more frequent in the physiological orientation, since highly negatively supercoiled topoisomers should be produced in larger amounts in this orientation, which was clearly not the case (Fig. 3, B and C, no RNases). The fact that
Regulation of R-loop Formation by DNA Topoisomerase

The formation of highly negatively supercoiled DNAs was completely abolished by RNase H and only partially by RNase A, as may suggest that nascent RNAs are involved in R-loop formation; and therefore that DNA gyrase can participate in R-loop elongation when transcription is arrested, as suggested previously (22). However, this is not true if, as stated above, the RNase A effect reflects the stability of the R-loop and not the presence of free RNAs. Our results also suggest that longer RNase A effect reflects the stability of the R-loop and not the presence of free RNAs. Our results also suggest that longer R-loops are being produced when DNA gyrase is present during active transcription, since hypernegatively supercoiled plasmid DNAs are then produced, whereas when DNA gyrase is added after the arrest of transcription, only highly negatively supercoiled DNAs are produced (compare the two-dimensional gel of Fig. 1 (left panel) with the two-dimensional gel of Fig. 3B (no RNases)). This suggests that extensive R-loop formation occurs only during active transcription in a 5’ to 3’ direction and in the presence of DNA gyrase, as previously suggested in the case of the pBR322 RNAII primer (26). In this context, the role of DNA gyrase is to counteract extensive relaxation of plasmid DNAs due to R-loop elongation and therefore to allow more extensive R-loop elongation. This is because in the absence of DNA gyrase, extensive R-loop elongation could eventually be blocked by the accumulation of positive supercoils.

_E. coli_ DNA Topoisomerase I Efficiently Abolishes Hypernegative Supercoiling and Hence R-looping during Transcription in the Presence of DNA Gyrase—Data presented above shows that the hypernegative supercoiling reaction in the presence of DNA gyrase was an efficient way of detecting R-loop formation during transcription. Therefore, this assay can be useful to evaluate the effects of DNA topoisomerase I on R-loop formation during active transcription. Previous _in vitro_ studies have shown that the relaxation activity of _E. coli_ DNA topoisomerase I was strongly activated by transcription (22, 27). This can best be explained by the twin domain model of transcription, where DNA topoisomerase I relaxes negative supercoiling generated behind the moving RNA polymerase. Therefore, it is possible that DNA topoisomerase I directly inhibits R-loop formation by relaxing the local negative supercoiling induced by transcription, if local negative supercoiling is important for R-loop formation. The stimulated relaxation activity of DNA topoisomerase I during transcription can also efficiently counteract the supercoiling activity of DNA gyrase to keep global negative supercoiling of plasmid DNAs below the level required for R-loop formation.

pJP459 DNA was thus transcribed _in vitro_ by T3 RNA polymerase in the presence of various ratios of _E. coli_ DNA topoisomerase I to _E. coli_ DNA gyrase. As shown in Fig. 4, 40 ng of DNA topoisomerase I was sufficient to almost completely inhibit hypernegative supercoiling of pJP459 DNA. 11 ng of RNase H had the same inhibitory effect (Fig. 4). Therefore, at a roughly 1:1:1 molar ratio of DNA template, DNA gyrase and DNA topoisomerase I or RNase H, hypernegative supercoiling was inhibited. This result suggests that under our experimental conditions, DNA topoisomerase I was as efficient as RNase H to inhibit hypernegative supercoiling and hence R-looping in the presence of a similar amount of DNA gyrase.

The fact that RNase A at a high concentration did not abolish hypernegative supercoiling (see above), and hence R-looping, suggests that local DNA supercoiling generated during RNA polymerase movement is not a key factor for this reaction, at least when a DNA template with a superhelical density of about −0.05 is used. This is because, according to the twin domain model, nascent RNA should be required to create a frictional drag to inhibit RNA polymerase rotation and hence to allow the generation of negative and positive supercoils during RNA polymerase movement. In agreement with the model, RNase A at a very low concentration has been shown to inhibit transcription-induced positive supercoiling in the presence of _E. coli_ DNA topoisomerase I (27). Therefore, adding RNase A at high concentrations during transcription in the presence of both DNA gyrase and topoisomerase I should favor the hypernegative supercoiling reaction, if the transcription-generated negative supercoiling is important to stimulate the relaxation activity of DNA topoisomerase I. This is demonstrated in Fig. 5, where DNA topoisomerase I and DNA gyrase where mixed together during transcription of pJP459 DNA by T3 RNA po-

---

*Fig. 2. Time course of hypernegative supercoiling of the template DNA during transcription in the presence of DNA gyrase.* A, transcription reactions using T7 RNA polymerase and pJP459 or pJP461 DNA were performed under the conditions described under “Experimental Procedures.” Reactions were scaled up to 200 μl, and DNA gyrase was added in the beginning of the reaction. DNA samples (25 μl) were taken at 0 min and thereafter every 2 min and mixed with a stop solution (25 μl) as described under “Experimental Procedures.” DNA samples were analyzed by one-dimensional agarose gel electrophoresis, and the gel was dried and used for in situ hybridization. Numbers above the lanes indicate the time (in min) when DNA samples were taken and mixed with a stop solution. B is A, except that RNase A at 20 μg/ml was added in the beginning of the reactions. [– –] indicates the position of hypernegatively supercoiled DNAs in the gel.
Fig. 3. Two-stage reactions showing the production of highly negatively supercoiled DNA in the presence of DNA gyrase and after the arrest of transcription. Transcription reactions using T3 or T7 RNA polymerase and pJP459 or pJP461 DNA were performed for 20 min under the conditions described under “Experimental Procedures.” Transcription was arrested by heating at 65°C for 20 min and RNases (RNase A (40 μg/ml); RNase H (20 ng)) were added or not as indicated above the lanes in A. After an incubation of 10 min at 37°C, DNA gyrase was added, and the samples were incubated for an additional 10 min before the addition of the stop solution. DNA samples were analyzed by one-dimensional agarose gel electrophoresis in A and by two-dimensional agarose gel electrophoresis in B and C. The gels were dried and used for in situ hybridization. In A, non-transcribed means that RNA polymerase was omitted in the reaction; [-] indicates the position of highly negatively supercoiled DNA in the gel; relaxed indicates the position of relaxed DNA under the chloroquine concentration used during electrophoresis. Some of the DNA samples analyzed in A were also analyzed in B (pJP459) and C (pJP461). T3, RNase H, RNase A, and no RNases, respectively, correspond to the same pJP459 (B) or pJP461 (C) lanes in A. T7, RNase H, RNase A, and no RNases, respectively, correspond to the same pJP459 (B) or pJP461 (C) lanes in A.

Regulation of R-loop Formation by DNA Topoisomerases
molecules were found to carry R-loops in this population (data not shown).

**DISCUSSION**

In the present report we have investigated the role of DNA topoisomerases in R-loop formation during transcription. Our previous results have indicated that the level of negative supercoiling was an important determinant of R-loop formation in vivo and in vitro. This is in agreement with earlier results (28–30). It means that one key role of DNA gyrase is to maintain the negative superhelicity at a level appropriate to allow R-loop formation. In agreement with this result, R-loop formation was not detected in our two-stage reaction when a relaxed DNA template was used during transcription (data not shown).

Once R-loop formation is initiated it can elongate if sufficient negative superhelicity is maintained throughout this process. In this context, on a small supercoiling domain such as the pJP459 and pJP461 DNAs used in this study, the role of DNA gyrase is predictable and was indeed observed. Therefore, DNA gyrase will maintain high negative superhelicity throughout the process of R-looping and will produce hypernegatively supercoiled plasmid DNAs upon R-loop removal by RNase H. However, this role of DNA gyrase in R-loop elongation might not be as important in a chromosomal DNA context if only very large supercoiling domains exist (31) and if the diffusion of the supercoils is fast enough. This is because in this context, extensive R-loop elongation will not significantly affect the negative supercoiling level of the domain. However, small supercoiling domains may at least transiently exist, due, for example, to the presence of moving transcription complexes and large DNA-bound protein complexes (20).

We have also investigated the role of *E. coli* DNA topoisomerase I in R-loop formation. We found that the strong transcription-induced relaxing activity of *E. coli* DNA topoisomerase I can inhibit R-looping, probably by modulating the global DNA supercoiling level of the DNA template. However, it is still possible that the transcription-induced negative supercoiling might be important for R-loop formation, since half of the global supercoiling is believed to be constrained in vivo by proteins binding to negatively supercoiled DNA (2). In this context, DNA topoisomerase I will control R-loop formation by modulating the transcription-induced negative supercoiling. In
addition, we found that the R-loop structure is a hot spot for relaxation by E. coli DNA topoisomerase I. Therefore, relaxation by this enzyme as soon as R-loop formation is initiated may result in the destabilization of the short R-loop. E. coli DNA topoisomerase I can thus indirectly prevent R-loop initiation and directly prevent R-loop elongation, at least in our in vitro system. In this context, it is easy to understand why this enzyme is a key element in the regulation of R-loop formation. Also, since R-loop formation can modulate both DNA gyrase and DNA topoisomerase I activity, it can possibly be an important element in the regulation of the global DNA supercoiling level in vivo. One can also predict that RNase H participates in this function.

We also used the hypernegative supercoiling reaction to investigate the mechanism of R-loop formation. Together, the fact that hypernegative supercoiling was only produced during active transcription and was, at least in some situations, resistant to the presence of RNase A at a high concentration, suggest that the RNA in the hybrid was never free and, therefore, that R-loop formation occurred in a 5’ to 3’ direction. A similar conclusion was previously reached when RNA primer formation during pBR322 transcription in the presence of DNA gyrase was studied (26). However, our results do not exclude the possibility that R-loop formation also occurs from nascent RNA in a 3’ to 5’ direction during active transcription in the absence of RNase A. Indeed, previous studies have suggested that R-loop formation during pBR322 transcription with E. coli RNA polymerase occurred by this mechanism (22). The fact that hypernegative supercoiling during transcription in the absence of RNase A was nonspecific but was specific in the presence of RNase A suggests that some R-loops are more stable, or persistent, than others. To be biologically meaningful, the stability factor is probably the most important for an R-loop. Therefore, R-loop formation during transcription of the rrrB HindIII fragment in the physiological orientation is possibly biologically significant. Indeed, we observed that transcription of this fragment, preferentially in its physiological orientation, on a plasmid DNA partially inhibited the growth of a topA mutant if RNase H was not overproduced. Also, our results suggest that unstable R-loops are frequently produced during transcription and that DNA gyrase, acting very rapidly, allows their detection. Considering that the RNase A effect reflects R-loop stability, it may suggest that our gel retardation experiments, in which the DNA samples were treated with RNase A at a high concentration prior to electrophoresis, can also be explained in this context. The fact that when a hypernegatively supercoiled plasmid DNA was used as a template during transcription in vitro (data not shown) and in vivo, R-loop formation was still specific to the physiological orientation of the rrrB fragment, as shown by the gel retardation assay, can also be explained in the context of R-loop stability. It may suggest that increasing negative supercoiling above a certain level does not significantly enhance R-loop stability.

Although the hypernegative supercoiling reaction can be a good assay to study R-loop formation in vitro, it may not be as useful to study this process in vivo. This is because so many factors can influence DNA supercoiling in vivo and because hypernegative supercoiling can possibly be generated in different ways in vivo (18, 21). One must also consider the possibility that in some situations hypernegative supercoiling occurs before R-loop formation and that the formation of such structures simply protects the hypernegatively supercoiled DNA from relaxation. In vivo, binding of histone-like proteins to DNA, for example, may also protect hypernegatively supercoiled DNA from being relaxed.

Acknowledgments—We thank Dr. James C. Wang and Dr. Leroy F. Liu for providing us with purified E. coli DNA gyrase and DNA topoisomerase I. We also thank Robert J. Crouch for pure E. coli RNase H and Sonia Broccoli for careful reading of the manuscript.

REFERENCES

1. Wang, J. C. (1996) Annu. Rev. Biochem. 65, 635–692
2. Drlica, K. (1992) Mol. Microbiol. 6, 425–433
3. Wang, J. C. (1971) J. Mol. Biol. 55, 523–533
4. Gellert, M., Mizuuchi, K., O’Hara, M. H., and Nash, H. A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3872–3876
5. Nitsis, J. L. (1994) Adv. Pharmacol. 29A, 103–134
6. Menzel, R., and Gellert, M. (1994) Adv. Pharmacol. 29A, 39–69
7. Fleischmann, G., Pfugfelder, G., Steiner, E. K., Javaherian, K., Howard, G. C., Wang, J. C., and Elgin, S. C. R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 6958–6962
8. Muller, M. T., Pfund, W. P., Mehta, V. B., and Trask, D. K. (1985) EMBO J. 4, 1237–1243
9. Gilmour, D. S., Pfugfelder, G., Wang, J. C., and Lis, J. T. (1986) Cell 44, 401–407
10. Rose, K. M., Szopa, J., Han, F.-S., Cheng, Y. C., Richter, A., and Scheer, U. (1988) Chromosome 96, 411–416
11. Zhang, H., Wang, J. C., and Liu, L. F. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 1060–1064
12. Stewart, A. F., Herrera, R. E., and Nordheim, A. (1990) Cell 60, 141–149
13. Kroeger, P. E., and Rowe, T. C. (1992) Biochemistry 31, 2492–2501
14. Pruss, G. J., and Drlica, K. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 8952–8956
15. Wu, H. Y., Shyy, S. H., Wang, J. C., and Liu, L. F. (1988) Cell 53, 433–440
16. Lockshon, D., and Morris, D. R. (1983) Nucleic Acids Res. 11, 2999–3016
17. Pruss, G. J. (1985) J. Mol. Biol. 185, 51–63
18. Lynch, A. S., and Wang, J. C. (1990) J. Bacteriol. 175, 1645–1655
19. Franco, R. J., and Drlica, K. (1989) J. Bacteriol. 171, 6573–6579
20. Liu, L. F., and Wang, J. C. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7024–7027
21. Drolet, M., Wu, H.-Y., and Liu, L. F. (1994) Adv. Pharmacol. 29A, 135–146
22. Drolet, M., Bi, X., and Liu, L. F. (1995) J. Biol. Chem. 270, 2068–2074
23. Drolet, M., Phoenix, P., Menzel, R., Mas, E., Liu, L. F., and Crouch, R. J. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3526–3530
24. Kirkegaard, K., and Wang, J. C. (1985) J. Mol. Biol. 185, 625–637
25. Sambriski, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Parada, C. A., and Marinos, K. J. (1989) J. Biol. Chem. 264, 15120–15129
27. Tsao, Y. P., Wu, H.-Y., and Liu, L. F. (1989) Cell 56, 111–118
28. Wang, J. C. (1974) J. Mol. Biol. 87, 797–816
29. Richardson, J. P. (1975) J. Mol. Biol. 98, 565–579
30. Champeux, J. J., and McConaughy, B. L. (1975) Biochemistry 14, 307–316
31. Sinden, R. R., and Pettijohn, D. E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 224–228