It has recently been shown that RNase H overproduction can partially compensate for the growth defect due to the absence of DNA topoisomerase I in Escherichia coli (Drolet, M., Phoenix, P., Menzel, R., Massé, E., Liu, L. F., and Crouch, R. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3526–3530). This result has suggested a model in which inhibitory R-loops occur during transcription in topA mutants. Results presented in this report further support this notion and demonstrate that transcription-induced supercoiling is involved in R-loop formation. First, we show that stable R-loop formation during in vitro transcription with E. coli RNA polymerase only occurs in the presence of DNA gyrase. Second, extensive R-loop formation in vivo, revealed by the production of RNase H-sensitive hypernegatively supercoiled plasmid DNAs, is observed under conditions where topA mutants fail to grow. Furthermore, we have demonstrated that the coupling of transcription and translation in bacteria is an efficient way of preventing R-loop formation.

The nascent RNA is normally displaced during the process of transcription to be translated or to participate in the process of translation. Shortly after the discovery that cellular DNA can be negatively supercoiled, it was suggested that the favorable free energy of such supercoiling should maintain the base pairing between the nascent RNA and the template DNA strand, and consequently, should interfere with the process of RNA displacement. Indeed, a direct correlation between the level of negative supercoiling and the length of the RNA-DNA hybrid (or R-loop, the template strand is paired with RNA, leaving the non-template strand unpaired) after transcription with Escherichia coli RNA polymerase was found (1). The formation of such hybrids was later shown to be due to the denaturing of transcribing RNA polymerases, and hence to the use of protein denaturing agents to stop the transcription reactions (2). These experiments have also demonstrated that the RNA polymerase possesses a putative “separator” function allowing the nascent RNA to be displaced as transcription proceeds, and therefore a function that counteracts the favorable free energy of negative supercoiling for RNA-DNA hybrid formation. In agreement with this notion are the results from several experiments revealing that the 9–12-base pair RNA-DNA hybrid within the RNA polymerase is positioned very close to the downstream edge of the 18-base pair open transcription bubble (3). Thus, extensive R-loop formation originating from the short hybrid within the transcription bubble does not normally occur during transcription.

However, the results of several in vitro and in vivo experiments have clearly shown that extensive R-loops can form during transcription on negatively supercoiled templates, and that both the formation and the length of such structures is modulated by DNA topoisomerases (4–7). These topoisomerases are, DNA gyrase, responsible for the introduction of negative supercoiling, and DNA topoisomerase I, responsible for the relaxation of negative supercoiling (reviewed in Ref. 8). Indeed, in one study (5), overproduction of RNase H, an enzyme degrading the RNA moiety of an R-loop, was shown to partially correct the growth defect of topA null mutants. In another series of experiments, R-loop formation during transcription of a portion of the rrnB operon, encoding for rRNAs, was shown to occur both in vitro and in vivo in the absence of DNA topoisomerase I (6, 7). How these results can be pieced together with the early observations described above is still unknown. One way to reconcile all these observations is to consider that the R-loop does not originate from the transcription bubble but is initiated by the reannealing of a portion of the nascent RNA with a complementary DNA template region behind the moving RNA polymerase. This, of course, requires that the nascent RNA be free and therefore not bound by ribosomes and that the corresponding DNA region behind the moving RNA polymerase be opened. DNA opening behind the moving RNA polymerase can be nucleotide sequence-dependent and can be promoted by negative supercoiling generated during transcription in the frame of the twin-domain model (9). According to this model, supercoiling can be generated during transcription elongation because of the difficulty for a moving transcription complex to rotate around the double helix. In this situation, domains of negative and positive supercoiling are transiently generated, respectively, behind and ahead of the moving transcription complex. In the absence of DNA topoisomerase I, the local negative supercoiled domain can build up, whereas the positive one can be removed by DNA gyrase. Interestingly, we have recently shown that severe growth inhibition in the absence of DNA topoisomerase I correlates with transcription-induced supercoiling (10). Because RNase H overproduction stimulates the growth of the topA null mutants used to demonstrate this correlation (5), we thought that R-loop formation generated during transcription might be due to negative supercoiling generated behind the moving RNA polymerase, which accumulates in the absence of DNA topoisomerase I. In this report, we present biochemical and genetic evidence supporting this hypothesis. Moreover, in agreement with the model for R-loop formation described above, we present evidence that the binding of ribosomes to nascent RNAs can inhibit R-loop formation.

**EXPERIMENTAL PROCEDURES**

*Plasmids—The two plasmid DNAs used in this study are pBR322 derivatives and have been described elsewhere (Refs. 10 and 11; also...*
see Fig. 3). Briefly, pBR322Δtet5 has a small deletion within the promoter of the tetA gene that considerably reduces its expression. In pBR322Δtet5 an HindIII-EcoRV deletion removed the 5’ portion of the tetA gene, so that the remaining tetA RNA is not translated but still produced. pEM001 and pEM003 are pACYC184 derivatives that, respectively, carry the wild-type rnahA gene or a mutated version of this gene (7).

**In Vitro Transcription Reactions**—Typical in vitro transcription reactions were performed as described previously (4, 6). 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 pBR322 DNA, 1 unit of E. coli RNA polymerase (Amersham Pharmacia Biotech) and, when specified, RNase A and E. coli RNase H were added at the indicated concentrations. The reactions were incubated at 37 °C for 3 min before the addition (or not) of reconstituted E. coli DNA gyrase (about 50 ng of each subunit) and then incubated for an additional 10 min at the same temperature. In Fig. 1, the reactions were terminated by the addition of EDTA to a final concentration of 30 mM. The samples were brought to 0.3 x NaCl final before the addition of 100 ng of RNase A, followed by a 60-min incubation period at 37 °C. The reactions were extracted with phenol once, chloroform once, and precipitated with ethanol. They were resuspended in 10 mM Tris (pH 8.0), 10 mM MgCl₂ and treated or not with 7.5 ng of RNase H for 60 min at 37 °C. The samples were analyzed by electrophoresis as indicated. In Fig. 2, the 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 30 min of incubation at 37 °C, the samples were extracted once with phenol, once with chloroform, and then precipitated with ethanol. They were resuspended in 10 mM Tris (pH 8.0), 10 mM MgCl₂, 100 mM NaCl, and treated with 1 µg of RNase A and 20 ng of RNase H. After 45 min of incubation at 37 °C, the samples were phenol-extracted and then analyzed by electrophoresis.

**Plasmid Extraction for Supercoiling Analysis**—For the extraction of plasmid DNAs for supercoiling analysis, the following procedure was used. RFM480 cells (topA20::Tn10, gyrB221(cou83), gyrB203(Ts), Ref. 5) carrying the various pBR322 derivatives were grown overnight in VB Cassa medium at 37 °C and then diluted 1/75 in prewarmed LB medium. All the media were supplemented with ampicillin at 50 µg/ml and chloramphenicol at 30 µg/ml when required. The cells were grown to an A₆₀₀ of 0.4 at 37 °C at which time they were transferred to the desired temperature. The plasmid DNAs were extracted when the A₆₀₀ reached about 0.7 (all the strains at 37 °C and only the ones carrying pEM001 at 25 °C and therefore overproducing RNase H at 28 °C and all the strains at 21 °C). When hypernegatively supercoiled plasmid DNAs were produced at 21 °C, the proportion of such topoisomers reached a maximum after about 1 h at this temperature and did not change for at least another hour (data not shown). Growth was stopped by transferring the cells in a tube filled with ice. With this procedure, the temperature of the cultures immediately dropped to 0 °C. Plasmid DNAs were extracted by an alkaline lysis procedure (12).

**Electrophoresis**—One-dimensional and two-dimensional agarose gel electrophoresis in the presence or absence of chloroquine were performed in 0.5 x TBE as described (7). After electrophoresis, the gels were either stained with ethidium bromide and photographed under UV light (Figs. 1 and 2) or dried and prepared for in situ hybridization (Figs. 4–6) as described (7).

**RESULTS**

**Extensive R-loop Formation during In Vitro Transcription with E. coli RNA Polymerase Occurs Only in the Presence of DNA Gyrase**—To study how DNA supercoiling affects R-loop formation during transcription with E. coli RNA polymerase, we performed in vitro transcription experiments with a supercoiled DNA template in the presence or absence of DNA gyrase. The DNA template used, pBR322, was extracted from a wild-type strain and therefore had a higher level of negative supercoiling than DNA from topA4 null mutants with gyr mutations. Moreover, its effective negative supercoiling density was significantly higher than DNA from wild-type cells, considering that about half of the DNA supercoiling is constrained in vitro (8). To detect R-loop formation, we used the previously described assay in which RNase H-sensitive gel retardation and/or relaxation of plasmid DNAs after transcription is revealed following electrophoresis and ethidium bromide staining of the gel (7). In addition, all the in vitro reactions were arrested with EDTA and treated with RNase A before being phenol-extracted. This procedure was used, because R-loop formation involving nascent RNA (sensitive to RNase A) can be induced following denaturing of E. coli RNA polymerases that transcribe DNA templates with a wild-type supercoiling level (Ref. 2 and data not shown). The results shown in Fig. 1 clearly demonstrate that significant and stable R-loop formation exclusively occurs when DNA gyrase is present during the transcription reaction. Indeed, RNase H-sensitive gel retardation of plasmid DNAs is only detected when DNA gyrase was added during transcription (compare lane 4, –RNase H with lane 5, +RNase H after transcription, lane 7, +RNase H during transcription or lane 8, +RNase H during and after transcription). This alteration in the electrophoretic mobility is better seen in the gel containing chloroquine (Fig. 1B, lane 4), because plasmid DNAs carrying R-loops are hypernegatively supercoiled (see below). Hypernegatively supercoiled plasmid DNA repre-
Escherichia coli RNA Polymerase in R-loop Formation

Fig. 2. Hypernegative supercoiling during in vitro transcription with E. coli RNA polymerase. The transcription reactions were performed as described under “Experimental Procedures.” When indicated, 7.5 ng of RNase H and/or 1 μg of RNase A were added during transcription. 3 NTPs means that GTP was omitted during transcription. pBR322 represents a sample of the plasmid DNA used in this study. The samples were analyzed by electrophoresis in an agarose gel containing 7.5 μg/ml of chloroquine.

resents a population of topoisomers that can no longer be resolved by electrophoresis in agarose gels containing chloroquine (11, 13). These in vitro results demonstrate that an effective global negative supercoiling level, even higher than the effective level existing in topA null mutants, is unable on its own to trigger the formation of stable R-loops. This is in agreement with the results of early experiments (2). Most likely, R-loop initiation during transcription by E. coli RNA polymerase involves transcription-induced supercoiling, and DNA gyrase participates in the process of R-loop elongation to generate stable and detectable R-loops.

We next wanted to study more precisely the link between hypernegative supercoiling and R-loop formation during in vitro transcription with E. coli RNA polymerase, in the presence of DNA gyrase. Fig. 2 shows the results of an experiment in which the susceptibility of hypernegative supercoiling formation to RNase A and/or RNase H treatments was evaluated. It can be seen that both RNase A and H dramatically reduced the production of hypernegatively supercoiled pBR322 DNA in the presence of DNA gyrase (compare lane 1, complete with lane 2, RNase H and lane 3, RNase A). Interestingly, RNase A and H together completely abolished hypernegative supercoiling (lane 6). This result may suggest that two independent mechanisms are operating to generate hypernegative supercoiling: one directly linked to the twin-domain model (sensitivity to RNase A) and the other one linked to R-loop formation independent of the twin-domain model (sensitivity to RNase H). However, by a more careful look at lanes 2 and 3, it is clearly revealed that both independent treatments to RNase A and H abolished more than half of the amount of hypernegatively supercoiled DNA generated in the absence of RNases (lane 1). Moreover, by increasing the amount of RNase H we found that it is possible to completely abolish hypernegative supercoiling (data not shown). Therefore, hypernegative supercoiling during transcription by E. coli RNA polymerase in the presence of DNA gyrase is completely dependent on R-loop formation, involving the participation of nascent RNA. In this context, the nascent RNA may serve two purposes: it is involved in generating negative supercoiling during transcription according to the twin-domain model, as previously shown in similar in vitro systems (4, 14), and it anneals with the complementary DNA strand to form the R-loop. This is in contrast with what has been demonstrated for hypernegative supercoiling during transcription with phage T3 and T7 RNA polymerases (6). Indeed, in these cases, the formation of such topoisomers was more sensitive to RNase H treatment but was highly resistant to RNase A treatment, suggesting that R-loop formation did not involve free RNA and occurred in the 5’ to 3’ direction, and therefore the newly synthesized RNA was never displaced from the template strand (6). It is worth mentioning that the natural DNA template for T3 and T7 RNA polymerases is not supercoiled but linear. Perhaps these polymerases do not possess an efficient RNA-DNA hybrid separator function as found for E. coli RNA polymerase, to counteract the favorable free energy for R-loop formation during transcription of a negatively supercoiled template. Indeed, stable R-loop formation on supercoiled templates during transcription by T3 and T7 RNA polymerases is detected in the absence of DNA gyrase, as opposed to the situation with E. coli RNA polymerase (Fig. 1, lane 3), and under the same experimental set-up as the one used in this study (7).

R-loop-dependent Hypernegative Supercoiling of Plasmid DNAs in topA Null Mutants Occurs When DNA Gyrase Is Very Active during Transcription and in the Absence of Translation—Our next goal was to reproduce the in vitro data presented above in vivo, and to test the model for R-loop formation involving free nascent RNA and transcription-induced supercoiling. Because R-loop formation can be induced on a negatively supercoiled template by the use of protein denaturing agents during nucleic acids extraction, we decided to use the RNase H-sensitive hypernegative supercoiling assay to reveal R-loop formation in vivo. The generation of such topoisomers occurs within the cells, and it is therefore a more reliable assay to reveal R-loop formation in vivo. Because all the previous in vitro and in vivo studies have shown that detectable R-loop formation does not occur in the presence of DNA topoisomerase I, our in vivo experiments were performed in a topA null mutant. In addition, because the presence of cellular RNase H could potentially be a problem, we used a topA null mutant that grows better when RNase H is overproduced (5). This topA null mutant, RFM480, carries the topA20::Tn10 allele and a gyrB(Ts) allele allowing the modulation of cell growth in a manner that depends on the temperature. Low temperatures (30°C and below) are more restrictive for this strain, owing to this gyrB(Ts) allele that represses a more wild-type level of activity under these conditions. This explains why the growth of RFM480 is cold-sensitive. At these temperatures, the topA null mutant behaves as a true topA mutant without compensatory mutations, and its growth is shown to be stimulated by overproducing RNase H (5).

The plasmid DNAs used in our studies are pBR322 derivatives. One derivative, pBR322Aptet (Fig. 3), has a small deletion within the tetA promoter region that was originally believed to abolish tetA gene expression (11). This plasmid was used to show that the formation of hypernegatively supercoiled pBR322 was linked to tetA gene expression, because such topoisomers were not detected when it was extracted from a widely used topA null mutant, DM800 (11). The formation of hypernegatively supercoiled pBR322 DNA was later shown to be due to membrane anchorage of the transcription complex via the tetA gene product, a membrane bound protein (15). We have recently found that pBR322Aptet confers low level tetracycline resistance, because a weak promoter was reconstituted during the construction of this plasmid. When this plasmid was ex-

1 E. Massé and M. Drolet, unpublished results.
tracted from our cold-sensitive topA null mutants exposed to nonpermissive temperatures, transcription-dependent hypernegatively supercoiled plasmid DNA was detected (10). This result provided evidence that severe growth inhibition of topA null mutants correlates with transcription-induced supercoiling but not with global supercoiling. We found that an EcoRI-EcoRV deletion within pBR322 that totally abolishes tetracycline resistance and tetA gene expression, also almost completely abolished the formation of such topoisomers (data not shown). This result suggests that the generation of hypernegatively supercoiled pBR322Δpet DNA is due to the residual tetA gene expression originating from that plasmid. We found that RNase H overproduction, conferred by the presence of the multicopy plasmid pEM001 that carries the rnhA gene, had no effect on the formation of such topoisomers (data not shown). This means that R-loop formation is not involved in hypernegatively supercoiling of pBR322Δpet DNA. According to our model for R-loop formation, this is not a surprising result, because the tetA mRNA from that plasmid is translated, and therefore the nascent RNA is not free to hybridize with the template DNA strand. Results presented below support this conclusion.

The other pBR322 derivative used in the present study is pBR322Δtet5′, from which the remaining portion of the tetA gene is transcribed but the resulting RNA is not translated (Fig. 3). This is because the HindIII-EcoRV deletion that was made to construct this plasmid eliminated the 5′ part of the tetA gene including the original −10 promoter region, the Shine-Dalgarno sequence (ribosome binding site), the ATG initiation codon, and one transmembrane domain responsible for the anchorage of the TetA protein to the membrane. However, an active promoter was reconstituted (Fig. 3). This promoter is almost as active as the original tetA promoter, according to lacZ assays (data not shown). When this plasmid DNA was extracted from our cold-sensitive topA null mutants exposed to nonpermissive temperatures, transcription-dependent hypernegatively supercoiled topoisomers were detected (10). The formation of such topoisomers is linked to tetA gene transcription, because, as mentioned above, a larger deletion, EcoRI-EcoRV (Fig. 3), which completely eliminates tetA gene transcription, also dramatically reduces the accumulation of such topoisomers. The next series of experiments was performed to verify if the production of hypernegatively supercoiled pBR322Δtet5′ DNA was linked to R-loop formation. For that purpose, we introduced an additional plasmid DNA, pEM001, carrying the rnhA gene or the control plasmid, pEM003, carrying an inactive rnhA gene, within RFM480 bearing pBR322Δtet5′. The cells were grown in LB medium at 37°C and exposed to the indicated temperatures as described under “Experimental Procedures.” The extracted plasmid DNAs were subjected toelectrophoresis in agarose gel in the presence of chloroquine at 7.5 μg/ml. Under these conditions the more negatively supercoiled topoisomers migrate slowly except for the fastest migrating band pointed out by an arrow (Fig. 4, [− −]), which represents hypernegatively supercooled plasmid DNAs. First of all, it can be seen that the global DNA supercoiling level in the various strains, represented by the topoisomers distributions of pBR322Δpet DNA without considering hypernegatively supercoiled DNA, increases as the temperature decreases. This is expected, because the temperature-sensitive DNA gyrase becomes more active at low temperatures. It is also obvious that RNase H overproduction did not have any effect on global supercoiling level (for example, compare lane 5, +pEM001 with lane 6, +pEM003). Results presented in Fig. 4 also clearly demonstrate that, as opposed to pBR322Δpet DNA, RNase H overproduction abolished the generation of hypernegatively supercoiled pBR322Δtet5′ DNA (compare lane 5 with lane 6, 28°C and lane 8 with lane 9, 21°C). Most likely, such topoisomers are not produced at 37°C, because DNA gyrase activity for R-loop elongation is too weak to counteract the wild-type level of RNase H activity. As the temperature decreases, DNA gyrase regains a higher level of activity, and the wild-type level of RNase H activity is no longer sufficient to completely abolish extensive R-loop formation; hence, the generation of hypernegatively supercoiled pBR322Δtet5′ DNA. Two-dimensional agarose gel analysis was also performed to confirm the presence of hypernegatively supercoiled pBR322Δtet5′ DNA and the fact that its formation is abolished by overproducing RNase H (Fig. 5, top panels). Fig. 5 also shows that RNase H overproduction partially abolished the formation of hypernegatively supercoiled pACYC184Δtet5′ (bottom panels), a pACYC184 derivative carrying an identical deletion to the one found in pBR322Δtet5′. The tetA gene is the only common DNA se-

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**FIG. 4.** The formation of RNase H-sensitive hypernegatively supercoiled plasmid DNAs in a cold-sensitive topA null mutant, one-dimensional gel analysis. RFM480 cells carrying pBR322Δtet5′ were grown, and the plasmid DNAs were extracted as described under “Experimental Procedures.” In lanes 1, 4, and 7, the cells carry no additional plasmid DNA, whereas in lanes 2, 5, and 8 they also carry pEM001, and in lanes 3, 6, and 9, they also carry pEM003. + indicates that RNase H was overproduced (the cells carrying pEM001). [− −] indicates hypernegatively supercoiled plasmid DNAs. The samples were analyzed by electrophoresis in an agarose gel containing 7.5 μg/ml of chloroquine. The gel was probed with a 32P-labeled DNA fragment carrying the bla gene of pBR322.

**FIG. 3.** The 5′ tetA gene region of the various pBR322 derivatives used in this study. The 5′ tetA region of A (pBR322), B (pBR322Δpet) and C (pBR322Δtet5′). −35 and −10 indicate the nucleotide sequence determinants for ς70 E. coli promoters; +1 refers to the transcription initiation site; RBS to the ribosome binding site (Shine-Dalgarno sequence); and ATG to the initiation codon for the TetA protein. The promoter activity of the tetA gene of pBR322Δtet5′ was confirmed when the lacZ gene was cloned downstream in the appropriate orientation.
the equivalents of pEM001 and pEM003 but contain a ColE1 origin of replication that is compatible with pACYC184Δtet5′ (5). The samples were analyzed by two-dimensional agarose gel electrophoresis. The chloroquine concentrations used were 7.5 μg/ml and 30 μg/ml, respectively, in the first and second dimension. Under the chloroquine concentrations used, hypernegatively supercoiled plasmid DNAs migrate at the end of the left part of the curve. When pBR322Δtet5′ was analyzed, the gel was probed with a 32P-labeled DNA fragment carrying the bla gene of pBR322. When pACYC184Δtet5′ was analyzed, the gel was probed with a 32P-labeled DNA fragment carrying the cat gene of pACYC184.

Fig. 5. The formation of RNase H-sensitive hypernegatively supercoiled plasmid DNAs in a cold-sensitive topA null mutant, two-dimensional gel analysis. RFM480 cells carrying either pBR322Δtet5′ (top panels) or pACYC184Δtet5′ (bottom panels) were grown, and the plasmid DNAs were extracted as described under “Experimental Procedures.” The cells also carry either pEM001 (top left panel), pEM003 (top right panel), pSK760 (bottom left panel), or pSK762c (bottom right panel). pSK760 and pSK762c are, respectively, the equivalents of pEM001 and pEM003 but contain a ColE1 origin of replication that is compatible with pACYC184Δtet5′ (5). The samples were analyzed by two-dimensional agarose gel electrophoresis. The chloroquine concentrations used were 7.5 μg/ml and 30 μg/ml, respectively, in the first and second dimension. Under the chloroquine concentrations used, hypernegatively supercoiled plasmid DNAs migrate at the end of the left part of the curve. When pBR322Δtet5′ was analyzed, the gel was probed with a 32P-labeled DNA fragment carrying the bla gene of pBR322. When pACYC184Δtet5′ was analyzed, the gel was probed with a 32P-labeled DNA fragment carrying the cat gene of pACYC184.

Fig. 6. Sensitivity of hypernegative supercoiling of pBR322 DNA to the protein synthesis inhibitor, spectinomycin. RFM480 cells carrying pBR322 were grown to an A600 of 0.4 at which time spectinomycin (500 μg/ml) was added (lanes 1 and 2) or not (lanes 3 and 4). The cells were incubated for an additional 15 min at 37 °C before being exposed to 21 °C. An aliquot of cells was rapidly withdrawn for plasmid DNA extraction (lanes 1 and 3). The cells were incubated for an additional 2 h before the second plasmid DNAs extraction (lanes 2 and 4). Note that the topoisomer distribution is more bimodal (hypernegative supercoiling and global supercoiling) when translation is inhibited (compare lane 2 with lane 4). This is observed when hypernegative supercoiling is R-loop-dependent (Fig. 4). When translation is not inhibited, the topoisomer distribution is more heterogeneous and continuous, as previously shown for pBR322 DNA extracted from various topA mutants (11, 15, 15).

One prediction that could be made regarding hypernegatively supercoiled pBR322 DNA, is that the formation of such topoisomers should be insensitive to protein synthesis inhibitors under conditions where DNA gyrase is active enough during transcription to promote R-loop formation. This is demonstrated by the experiment shown in Fig. 6. Indeed, the protein synthesis inhibitor spectinomycin abolished the formation of hypernegatively supercoiled pBR322 only under conditions where DNA gyrase was not very active, therefore when RFM480 cells were grown at 37 °C (Fig. 6, compare lane 1 with lane 3, respectively, + or − spectinomycin). Under such conditions, hypernegative supercoiling has been shown to be dependent on membrane anchorage of the transcription complex via the TetA protein (15). However, when RFM480 cells were exposed to 21 °C, hypernegative supercoiling was not abolished by spectinomycin treatment as expected, because at this temperature DNA gyrase is active enough to promote R-loop-dependent hypernegative supercoiling (Fig. 6, compare lane 2 with lane 4, respectively, + or − spectinomycin). When a similar experiment was performed with the pBR322 derivative carrying the EcoRI-EcoRV deletion that completely inactivates tetA gene expression, only a very small amount of hypernegatively supercoiled plasmid DNAs could be detected (data not shown). This result suggests that R-loop-dependent hypernegative supercoiling of pBR322 in the absence of translation is mostly related to tetA gene transcription.

All together, the results of our in vivo experiments are in accordance with the model for R-loop formation during transcription by E. coli RNA polymerase. 1) RNase H-sensitive hypernegatively supercoiled plasmid DNA, and hence R-loop formation, is detected under conditions where DNA gyrase is very active during transcription. Indeed, under these conditions, very weak tetA gene expression from pBR322ΔPtet DNA is still sufficient to trigger the formation of hypernegatively supercoiled plasmid DNA. 2) It is only in the absence of translation that RNase H-sensitive hypernegatively supercoiled plasmid DNA, and hence R-loop formation, is detected. This is also in agreement with our previous results showing RNase H-sensitive hypernegative supercoiling when a portion of the untranslated rrnB operon was transcribed (7).

DISCUSSION

Two major conclusions emerge from the work presented here. First, R-loop formation is linked to transcription-induced supercoiling but not to global supercoiling level. This is in agreement with the results demonstrating that extensive R-loop formation does not normally occur during transcription on negatively supercoiled templates unless E. coli RNA polymerase is denatured (Ref. 2 and data not shown). This result shows that even under conditions where the negative supercoiling level favors R-loop formation, they do not form. It may suggest that DNA supercoiling is not a major contributor to R-loop formation when such structures are generated. The best studied example of R-loop formation involves the origin of replication of the ColE1 plasmid DNA. In these studies, R-loop formation was clearly shown to be nucleotide sequence-dependent (16, 17). However, the results of several in vitro and in vivo experiments, including the work presented here, have clearly shown that extensive R-loops can form during transcription and that it is regulated by DNA topoisomerases that modulate the supercoiling level in E. coli (4–7). Because the global supercoiling level does not seem to be involved in R-loop formation, one obvious alternative explanation for the contribution of...
DNA topoisomerases in this process is in relation to transcription-induced supercoiling in the frame of the twin-domain model (9). Our results support this interpretation. The model derived from this interpretation implies that the free nascent RNA, in addition to being directly involved in RNA-DNA hybrid formation, also contributes to the generation of negative supercoiling during transcription. Such supercoiling can promote DNA opening behind the moving RNA polymerase, which is a prerequisite to the initiation of the annealing between the nascent RNA and the corresponding DNA template region. DNA opening could also be promoted by specific DNA sequences and/or global supercoiling level. Once the R-loop is initiated, it creates an anchor for the moving RNA polymerase, inhibiting its rotation and hence increasing transcription-induced negative supercoiling. This supercoiling can promote R-loop elongation if it is not relaxed by DNA topoisomerase I. Indeed, anchorage of the RNA polymerase via the annealing of the nascent RNA with the DNA template strand was originally described as one potential mechanism for increasing transcription-induced supercoiling (9). Anchoring the RNA polymerase will also increase transcription-induced positive supercoiling. Such supercoiling must be removed in order for the RNA polymerase and R-loop extension to progress at a proper rate. DNA gyrase will be involved in relaxing positive supercoiling, and therefore will contribute to R-loop elongation. DNA gyrase can also promote R-loop elongation by constantly replacing the negative supercoils removed by this process. Either way, DNA gyrase must be active enough to counteract the cellular RNase H activity that disrupts the R-loop. Therefore, if DNA gyrase is active enough, extensive, stable, and detectable R-loops will be generated. In the absence of RNase H, a mutated DNA gyrase, which even causes a decrease in global negative supercoiling below the wild-type level, will be sufficient for R-loop elongation. This is supported by the fact that double topA-rnhA mutants are nonviable (5) even when the strain carries very good compensatory gyr mutations, as in the case for the widely used DM800 topA null strain. Moreover, the growth of RFM480 in rich media is stimulated by RNase H overproduction even when global negative supercoiling is below the wild-type level (growth at 37 °C) (5). Interestingly, in vitro transcription experiments with E. coli RNA polymerase from synthetic RNA-DNA bubble duplexes have shown that the nascent RNA was frequently rehybridizing to the permanently unpaired DNA bubble (18). In the frame of our model for R-loop formation, the presence of this permanent bubble can be viewed as an optimal condition for R-loop initiation.

An important question that was not addressed in this study is related to RNA swiveling, which is required for extensive R-loop formation. In fact, the length of the R-loop when the RNA polymerase is still present on the template may not be limited by energetic considerations related to negative supercoiling, but rather by the capacity of the RNA to swivel. It is possible that the initiated R-loop behind the moving RNA polymerase eventually extends up to the transcription bubble. Under these conditions, the separator function of the RNA polymerase might be disrupted. In this context, swiveling of the RNA-DNA hybrid within the transcription bubble should be sufficient to allow the progression of transcription and R-loop elongation. This model can also explain the synthesis of the RNA primer at the CoE1 origin of replication (17). However, additional experiments are required to solve this problem.

The second important conclusion emerging from this study concerns the role of DNA topoisomerase I in E. coli. In a previous study (10), it was shown that severe growth inhibition of topA null mutants correlates with transcription-induced supercoiling. Together with the results presented in this study and the ones showing that RNase H overproduction stimulates the growth of topA null mutants (5), we can conclude that one major function of DNA topoisomerase I is to relax transcription-induced negative supercoiling to inhibit R-loop formation.

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