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THE PROBLEM OF HYPERNEGATIVE SUPERCOILING AND R-LOOP FORMATION IN TRANSCRIPTION

Marc Drolet, Sonia Broccoli, Fabien Rallu, Chadi Hraiky, Charles Fortin, Éric Massé and Imad Baaklini

Département de microbiologie et immunologie, Université de Montréal, C.P. 6128, Succ. Centre-ville, Montréal, P.Québec, Canada, H3C 3J7

TABLE OF CONTENTS

1. ABSTRACT

DNA supercoiling and topoisomerases have long been known to affect transcription initiation. In many studies, *topA* mutants were used to perturb chromosomal supercoiling. Although such studies clearly revealed that supercoiling could significantly affect gene expression, they did not tell much about the essential function(s) of DNA topoisomerase I, encoded by *topA*. Indeed, the *topA* mutants used in these studies were growing relatively well, although this gene is normally essential for growth. These mutants were either carrying a *topA* allele with enough residual activity to permit growth, or if deleted for the *topA* gene, they were carrying a compensatory mutation allowing them to grow. We have recently used a set of isogenic strains carrying a conditional *gyrB* mutation that allowed us to study the real effects of losing topoisomerase I activity on cell physiology. The results of our work show that an essential function of topoisomerase I is related to transcription, more precisely to inhibit R-loop formation. This is in agreement with a series of biochemical studies that revealed a role for topoisomerase I in inhibiting R-loop formation during transcription in the presence of DNA gyrase. In addition, our studies may have revealed an important role for DNA supercoiling in modulating gene expression, not only at the level of transcription initiation but also during elongation. In this paper, we will first discuss global and local supercoiling, then we will address the topic of R-loop formation and finally, we will review the subject of hypersupercoiling and R-loop formation in gene expression. Whenever possible, we will try to make correlations with growth phenotypes, since such correlations reveal the essential function of DNA topoisomerase I.

2. INTRODUCTION

The effect of DNA supercoiling on gene expression in *E. coli* has been the subject of many studies (reviewed in 1-3). Topoisomerase mutants were generally employed as tools to alter the global supercoiling level (for example see ref. 4), and less often they have been used to directly test for their involvement in the regulation of gene expression. This is particularly true for DNA topoisomerase I of *E. coli*, encoded by *topA*, but compensatory mutations must occur to allow these *topA* mutants to grow (5). Under such circumstances, it is difficult to attribute phenotypes to *topA* functions because, first of all, the presence of such compensatory mutations will correct the major phenotypes and therefore mask the key functions of this enzyme and second, phenotypes could be attributed to the compensatory mutation or to a combination of *topA* and the compensatory mutation. Nevertheless, enough data were obtained to predict that a major role of *topA* is to prevent excessive negative supercoiling (6), but not enough data was obtained to understand how such supercoiling will affect cell physiology and therefore why the absence of DNA topoisomerase I inhibits growth. Although *E. coli* DNA topoisomerase I was the first DNA topoisomerase to be discovered (7) and has been extensively studied biochemically (reviewed in 8-10), it is only recently that new data have been obtained about the precise cellular roles of this enzyme and, as a consequence, how excessive negative supercoiling could affect cell physiology. First, by studying the regulatory region of the *topA* gene, promoters were characterized under the control of sigma factors involved in response to several stress (11-12). This allowed to design experiments that demonstrated the involvement of *topA* in the response to heat-shock and oxidative stress (13-14). The other important advance was realized by using a

set of isogenic strains to test for the true effects of losing *topA* function on cell physiology (15). From these studies new important data were gained on not only the major functions of DNA topoisomerase I, but also on how DNA supercoiling can affect gene expression. According to this work, a major function of DNA topoisomerase I is to prevent hypernegative supercoiling and one of its major consequences, R-loop formation. The results of such studies should also contribute to our understanding of the role of *topA* in bacterial stress.

3. DNA TOPOISOMERASE I AND THE REGULATION OF GLOBAL AND LOCAL DNA SUPERCOILING IN *E. COLI*

3. 1. Global supercoiling

Based on genetic evidence, it has been postulated that the maintenance of a global level of chromosomal negative supercoiling within a +/- 15% range, is required for good growth of *Escherichia coli* cells (6). The global level of DNA supercoiling reflects the average superhelical density of all supercoiling domains. In this context, the essential function of DNA topoisomerase I, a member of the type IA family of topoisomerases, is to prevent chromosomal DNA from reaching an excessive level of negative supercoiling. This model stems from the observation that *topA* null mutants can grow significantly only if they accumulate compensatory mutations that are very often found in one of the genes encoding a subunit of DNA gyrase. As a result, global negative supercoiling of both chromosomal and plasmid DNA is decreased below the normal level (5, 16). Therefore, this global level of negative supercoiling is believed to be regulated by the opposing enzymatic activities of DNA topoisomerase I, encoded by the *topA* gene, that specifically relaxes negative supercoiling, and DNA gyrase, with two different subunits encoded by *gyrA* and *gyrB*, that introduces negative supercoiling (for reviews on DNA topoisomerases see ref. 8 and 10). However, although an increase in global negative supercoiling may perturb growth and affect many DNA transactions, recent data suggest that it does not explain the almost complete inhibition of cell growth in the absence of *topA* (see section 3.3).

3.2. Local supercoiling

The substrate specificity of DNA topoisomerase I for negatively supercoiled DNA has been explained by the fact that this topoisomerase binds to single-stranded DNA regions close to double-stranded ones (8, 9, 17). An appropriate level of negative supercoiling within the chromosomal DNA can promote the formation of such structures, since negative supercoiling favors DNA opening. Although readily relaxed by DNA topoisomerase I, a circular DNA molecule with a wild-type supercoiling level is not a hot-spot for relaxation by this enzyme. The process of transcription elongation, in the frame of the twin-domain model, can provide a hot-spot for DNA topoisomerase I activity. According to this model, domains of negative and positive supercoiling are transiently generated, respectively, behind and ahead of the moving transcription complex (18). Such local supercoiling can be generated during transcription elongation because of the difficulty for a moving transcription complex to rotate around the double helix. Although transient in nature as opposed to global supercoiling, this local supercoiling reaches much greater levels than global supercoiling and it is therefore subject to important fluctuations. The twin-domain model has been supported by much experimental evidence, which has also clearly implicated the process of transcription elongation in the generation of hot-spots for DNA topoisomerase I activity (19-22). In the absence of DNA topoisomerase I, the local negative supercoiled domain (negative hypersupercoiling) can build up, whereas the positive one can be removed by DNA gyrase. In some cases, especially when the transcribed gene encodes a membrane-bound protein, such as the *tetA* gene of pBR322, extreme negative supercoiling is generated by transcription (23, 24). Indeed, in this situation, the rotation of the RNA polymerase (RNAP) complex is completely inhibited, because of its anchorage to the membrane during coupled transcription-translation. When such genes are present on a plasmid DNA, transcription in *topA* null mutants has been shown to generate hypernegatively supercoiled DNA (22, 25). Hypernegatively supercoiled plasmid DNA represents a population of topoisomers that can no longer be resolved by electrophoresis in agarose gels containing chloroquine (22, 25, 26). Therefore, in the context of transcription elongation, the major role of DNA topoisomerase I is to control important local fluctuations of negative supercoiling, as opposed to simply maintaining global chromosome supercoiling at a constant level. Given the fact that in all these studies the experiments were performed with *topA* null mutants with compensatory gyrase mutations allowing them to grow, one may conclude that the removal of transcription-induced negative supercoiling by DNA topoisomerase I is not essential for cell growth. Therefore, despite the fact that the twin-domain model for transcription has been established as a concept, its physiological significance in *topA* mutants has remained obscure. However, a number of studies reveal that such supercoiling fluctuations due to transcription can also occur in wild-type cells (27, 28) and have physiological consequences (29, 30).

3.3. Global and transcription-induced supercoiling in growth inhibition of *topA* null mutants

We have recently used a set of *topA* null strains with different growth capabilities in combination with pBR322 derivatives to establish a correlation between growth defects and global or local (transcription-induced) supercoiling. With this system, we were able to show that severe growth inhibition in the absence of DNA topoisomerase I correlates with transcription-induced supercoiling, as shown by the accumulation of transcription-dependant hypernegatively supercoiled plasmid DNA, but not with global supercoiling (31). We concluded that the increase in the global level of negative supercoiling in some *topA* mutants is only a secondary consequence of the absence of DNA topoisomerase I and is not linked to the essential function of this enzyme. This is also in agreement with early observations that the nonsense mutation *topA10* with slight residual topoisomerase I activity on global supercoiling *in vitro*, does not inhibit growth despite the fact that global negative supercoiling is significantly higher in this strain as compared to an

isogenic wild-type strain (16). Moreover, almost no hypernegatively supercoiled pBR322 can be extracted from this strain when a *gyrB* compensatory mutation which reduces chromosomal supercoiling below the wild-type level is also present. However, a large amount of such pBR322 topoisomers can be extracted from a strain carrying a complete deletion of *topA* and a similar *gyrB* mutation (26). This result suggests that the *topA10* allele provides sufficient residual topoisomerase I activity for the removal of transcription-induced negative supercoiling, and explains why a cell carrying it can grow without the need for a compensatory mutation. Also in agreement with the fact that the essential function of *topA* is related to transcription, is our finding that multicopy suppressors of *topA* null mutations, such as RNase H (15, 32) and topoisomerase III (33), do not affect the global supercoiling level. The *topB* gene encodes DNA topoisomerase III, the other known member of the type IA family of topoisomerases in *Escherichia coli* (9). DNA topoisomerase III does not relax negatively supercoiled DNA with a wild-type superhelical density at physiological temperatures. This is probably because DNA topoisomerase III has a very high requirement, higher than that of DNA topoisomerase I, for single-stranded DNA for its activity (34). We have found that DNA topoisomerase III can relax transcription-induced negative supercoiling both *in vitro* and *in vivo.* In conclusion, our results reveal that the essential function of DNA topoisomerase I is linked to transcription-induced negative supercoiling. The major role of DNA topoisomerase I in controlling transcription-induced supercoiling was also shown functionally by studying the activity of the mutant *leu-500* promoter (35).

4. R-LOOP FORMATION AND HYPERNEGATIVE SUPERCOILING

4.1. Biochemical and genetic evidence for R-loop formation in the absence of DNA topoisomerase I

A good substrate for relaxation by DNA topoisomerase I can also be provided by an R-loop. In such a structure, the RNA is hybridized with the corresponding DNA template region, leaving the non-template strand unpaired. In this manner, single-stranded DNA regions close to double-stranded ones are generated. The results of *in vitro* experiments have shown that an R-loop can indeed be a hot-spot for relaxation by DNA topoisomerase I (36) and DNA topoisomerase III (33). The involvement of DNA topoisomerase I in the inhibition of R-loop formation was also suggested from the results of experiments in which this enzyme was shown to be required to maintain specificity in the process of initiation at *oriC* and ColE1 origins of replication *in vitro* (37, 38). RNA polymerase and DNA gyrase were also present in these *in vitro* replication assays. The interpretation was that DNA topoisomerase I was required to inhibit the formation of RNA-DNA hybrids at sites other than the normal origins of replication. The first indication that inhibition of R-loop formation is an important function of DNA topoisomerase I *in vivo* was obtained from studies done in our laboratory. Indeed, it was shown that the growth problem of *topA* null mutants can be partially corrected by overproducing RNase H, an enzyme that degrades the RNA moiety of an R-loop (15). A correlation was found between the level of DNA gyrase activity and the amount of RNase H required to rescue the growth of *topA* null mutants. The fact that *topA* null mutants acquire compensatory *gyr* mutations (5) was therefore explained by the supercoiling activity of DNA gyrase that promotes R-loop formation (15). In contrast, DNA topoisomerase I acts by inhibiting R-loop formation. This model, in which DNA topoisomerases with opposing enzymatic activities regulate the formation of growth-inhibitory R-loops, has been supported by results of *in vitro* experiments from our laboratory (19, 36, 39) and very recently confirmed by another laboratory (40). In these assays, R-loop formation was detected when DNA gyrase was present in sufficient excess over DNA topoisomerase I. R-loop formation was revealed by the formation of RNase H-sensitive hypernegatively supercoiled plasmids and/or R-looped plasmids, as shown by RNase H-sensitive gel retardation and relaxation. Using the same assays, we were able to detect R-loop formation on plasmids from *topA* null mutants (39). However, since R-loop formation has not yet been directly demonstrated *in vitro* and in *topA* null mutants, we cannot discriminate between several possibilities such as a very long R-loop, multiple very short R-loops or a single small R-loop from a stalled RNA polymerase that causes a piling-up of upstream transcribing RNA polymerases, to explain RNase H-sensitive hypernegative supercoiling of plasmids. Obviously, more *in vitro* and *in vivo* experiments need to be done to address this very important question. It is also worth mentioning that by using a topological assay, R-loops were revealed on pBR322 extracted from a *topA* null mutant (19). In agreement with the presence of R-loops on pBR322, hypernegative supercoiling due to *tetA* transcription was shown to be largely constrained in a *topA* null mutant as measured by Z-DNA formation (41).

More recently, we have demonstrated a correlation between R-loop formation during transcription and growth inhibition of *topA* null mutants (32). This established that the positive effect of RNase H overproduction on the growth of these mutants is linked to the inhibition of R-loop formation during transcription. The results of these experiments also suggests that transcription-induced negative supercoiling, not global supercoiling, might be responsible for the initiation of R-loop formation. The finding that the *rnhA* gene (encoding RNase H), normally a non-essential gene, is required for the growth of *topA* null mutants even when chromosomal negative supercoiling is below the wild-type level (42), supports this conclusion. Also supporting this conclusion, is the observation that topoisomerase III, an enzyme unable to relax global supercoiling but capable of stimulating the growth of *topA* null mutants when overproduced, can inhibit R-loop formation (33). In addition to local supercoiling, it is possible that a reduction of global negative supercoiling below the wild-type level is required in the absence of DNA topoisomerase I to avoid exceeding the capacity of the wild-type level of RNase H to remove inhibitory R-loops. Consistent with this hypothesis is the finding that a mutation in gyrase that reduces global negative supercoiling below the wild-type level, considerably improves the growth of *rnhA* mutants (15). Therefore, gyrase can apparently plays multiple roles in R-

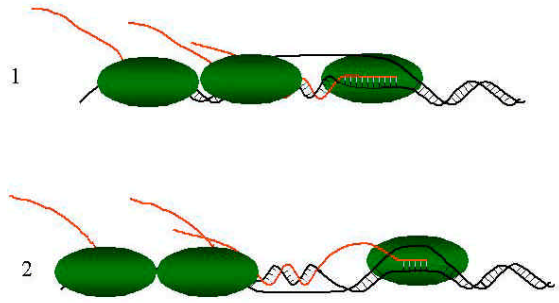

Figure 1. Models for the mechanism of R-loop formation and the inhibition of transcription. In 1, the R-loop is an extension of the RNA-DNA hybrid within the transcription bubble. In 2, the R-loop initiates by the reannealing of the nascent RNA with the template strand behind the RNA polymerase. In both cases, R-loop formation is stimulated by either global or local negative supercoiling and causes a piling-up of RNA polymerases.

loop formation, both at the nucleation and elongation steps, and both via global and local supercoiling. Finally, all these results suggest that a major role of DNA topoisomerase I is to inhibit R-loop formation by either reducing negative supercoiling to prevent its formation, or by acting as soon as the R-loop is initiated to destabilize it.

4.2. Mechanisms of R-loop formation

Since R-loop formation has not yet been directly demonstrated and therefore not sized and positioned relative to the RNA polymerase from which it originates, crucial information is still lacking and, consequently, it is not possible to completely understand the mechanism(s) of R-loop formation in *topA* mutants. A key question that should be addressed is related to the origin of the R-loop: does R-loop formation initiate from the transcription bubble (figure 1, top) and therefore are R-loops simply the extension of already existing 8-9 bp long RNA-DNA hybrids within RNA polymerase bubbles (43), or do R-loops initiate behind the moving RNA polymerase by the re-annealing of the nascent RNA with the template DNA strand (figure 1, bottom)? This is a very important question, because the first mechanism is directly linked to the mechanism of transcription, whereas the second one is not. Whatever the mechanism, it must take into account that the R-loop is accessible to RNase H which is normally not the case for the RNA-DNA hybrid within the transcription bubble. This is because all our experimental evidence for R-loops in *topA* mutants are linked to RNase H sensitivity and common phenotypes are shared by *topA* and *rnhA* mutants (see section 5). Here, we will review the available data that allows us to conclude that both mechanisms, not mutually exclusive, can in fact take place.

4.2.1. R-loop initiation from the transcription bubble

Normally, the nascent RNA is displaced from the template strand during transcription in order to be translated or to participate in the process of translation as tRNAs or rRNAs. Following the finding that cellular DNA can be negatively supercoiled, it was suggested that the favorable free energy of such supercoiling should preserve the base pairing between the nascent RNA and the template DNA strand and, as a result, should interfere with the process of RNA displacement. Indeed, a direct correlation between the level of negative supercoiling and the length of the R-loop after transcription with *Escherichia coli* RNA polymerase was observed (44). R-loop formation 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 (45). Within the limit imposed by the experimental approach used, RNA-DNA hybrids no longer than 20 bp were shown to exist when transcription was arrested with EDTA (a non-denaturing agent). These experiments have strongly suggested 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 R-loop formation. In agreement with this notion were the results from experiments revealing that a 8-9 bp RNA-DNA hybrid within the RNA polymerase is positioned very close to the downstream edge of a 18 bp open transcription bubble (46). Therefore, according to this model, since roughly 9 bp are opened behind the RNA-DNA hybrid, re-annealing of the template strand with the non-template strand, influenced by the level of negative supercoiling, is unlikely to be involved in the process of RNA displacement.

However, the results of other *in vivo* and *in vitro* experiments reveal that the size of the bubble can be very close to the size of the RNA-DNA hybrid. In such a case, re-annealing of the template strand with the non-template strand and, therefore, negative supercoiling, may participate in RNA displacement and, as a consequence, may dictate the size of the RNA-DNA hybrid. In one set of experiments, DNA footprinting with chemicals was used to probe the nucleic acids within a stalled RNA polymerase in *E. coli* (47). A bubble and an RNA-DNA hybrid of, respectively, 10-12 and 8-10 nucleotides in length were observed. The second set of experiments was performed with T7 RNA polymerase. Although this single subunit RNA polymerase is very different in amino acid sequence from the multi-subunit RNA polymerases such as the one from *E. coli*, it is mechanistically very similar at every step of transcription, namely initiation (48) elongation and termination (49 and references therein). In the first series of experiments with T7 RNA polymerase, enzymatic and chemical footprinting of stalled transcription complexes revealed a bubble of approximately 9 bp and an RNA-DNA hybrid of 7-8 bp in size (50). In another set of experiments, it was shown that T7 RNA polymerase elongation complexes can exist in two different states on negatively supercoiled templates: a state in which the RNA is displaced from the DNA and interacts with the RNA polymerase, and a state in which a more extended and

RNase H-sensitive hybrid is present and the RNA polymerase-RNA interaction is weaker (51). Partition between these two states was shown to be a function of the energy of template re-annealing which is dictated by the nucleotide sequence and negative supercoiling, and the relative strength of the RNA polymerase-RNA interaction and the strength of the RNA-DNA hybrid. Therefore re-annealing of the template strand with the non-template strand was clearly shown to be an important factor in RNA displacement. Moreover, the size of the transcription bubble was suggested to vary during transcription elongation, depending on the supercoiling level that fluctuates according to the twin-domain model. All these observations on transcription elongation with T7 RNA polymerase may explain the RNase H-sensitive hypernegative supercoiling *in vitro* and in *topA* null mutants in the following way: when the level of either local or global negative supercoiling increases due to the absence of topoisomerase I, an extended RNA-DNA hybrid, accessible to RNase H, forms within an RNA polymerase. This causes transcriptional arrest (see section 5), piling-up of RNA polymerases, extensive relaxation of the plasmid which is compensated for by the supercoiling activity of DNA gyrase and, finally, upon DNA extraction which removes the RNA polymerases on the plasmid, hypernegative supercoiling is revealed.

Our results with T7 RNA polymerase have also allowed us to conclude that R-loop formation originates from the transcription bubble (36, 39). Indeed, we have found that R-loop formation either illustrated by RNase H-sensitive hypernegative supercoiling or gel retardation of R-looped DNA, is highly sensitive to RNase H but very resistant to high amount of RNases that are specific to single-stranded RNA (RNase A and T). This means that the RNA in the R-loop was never displaced and therefore that the R-loop originates from the transcription bubble. Interestingly, in these experiments, although some sort of nucleotide sequence specificity was observed, the major determinant of R-loop formation was shown to be DNA supercoiling. When similar experiments were conducted with *E. coli* RNA polymerase, different results were obtained (32). Indeed, as opposed to T7 RNA polymerase, R-loop formation with *E. coli* RNA polymerase was found to be more resistant to RNase H and much more sensitive to RNases that are specific to single-stranded RNA. In fact, a much higher amount of RNase H was required to completely abolish R-loop formation. It was therefore concluded that for *E. coli* RNA polymerase, nascent RNA is involved in R-loop formation. In that case, the second model in which the nascent RNA behind the moving RNA polymerase re-anneals with the template strand (see section 4.2.2) seems more appropriate to explain R-loop formation with *E. coli* RNA polymerase. However, it is still possible to invoke the first model of R-loop formation for *E. coli* RNA polymerase, by simply considering that a higher negative supercoiling level is required to form an extended hybrid within this polymerase. Such supercoiling might be provided in the frame of the twin-domain model. This could also explain the sensitivity to single-stranded specific RNases, although the amount of such RNases required to eliminate R-loop formation is much higher than the amount used previously to abolish transcription-induced supercoiling *in vitro* (21 and see below for an alternative explanation). In agreement with this hypothesis is our finding that R-loop formation on supercoiled template is observed only when DNA gyrase is present during the reaction with *E. coli* RNA polymerase as opposed to T7 RNA polymerase. Presumably, DNA gyrase further increases the supercoiling level via the twin-domain model or simply via global supercoiling. The higher resistance to RNase H may be explained by a reduced accessibility of the extended hybrid to RNase H for *E. coli* RNA polymerase or, alternatively, to a hybrid with a different structure that renders it more resistant to RNase H and, at the same time, more sensitive to single-strand specific RNases. This would also explain the higher sensitivity of R-loop formation with *E. coli* RNA polymerase to such RNases and, at the same time, reconcile all the observations with the first model of R-loop formation. The high resistance of an extended hybrid within an *E. coli* RNA polymerase can also explain why RNase H needs to be overproduced to rescue the growth of *topA* null mutants, whereas only a fraction (less than 0.1%) of its activity is required to process the R-loop at the ColE1 origin of replication (52).

Also in agreement with the first model and therefore the fact that the mechanism of transcription is directly involved in R-loop formation, is the finding that two RNA polymerase mutations, *rpoB8* and *rpoB3595*, with opposing effects on transcription velocity, pausing and termination (53), have divergent effects on the growth of *topA* null mutants (15). One of them, *rpoB8*, which improves growth, also alleviates other phenotypes, including transcription-induced hypernegative supercoiling of plasmids (Hraiky *et al.*, unpublished results). Interestingly, the *rpoB8* mutation makes the RNA polymerase move slowly which should reduce transcription-induced supercoiling.

4.2.2. R-loop initiation behind the moving RNA polymerase

The second proposed model for the mechanism of R-loop formation supposes that it is not linked to the mechanism of transcription, since the nascent RNA behind the moving RNA polymerase initiates R-loop formation. Requirements for this mechanism are that the RNA behind the moving polymerase be free and therefore not bound by ribosomes (see section 4.3) and also that the DNA template be opened behind this moving polymerase. This second requirement could be met by invoking the twin-domain model and therefore, the accumulation of transient negative supercoiling behind the moving RNA polymerase in the absence of DNA topoisomerase I, which will promote DNA opening. In fact, this model is supported by the results of *in vitro* transcription experiments that were not originally designed to test it (54). In this series of experiments, Daube and von Hippel were studying RNA displacement from the RNA polymerase during transcription, after initiation from a synthetic permanent bubble. Because of base mismatches between template and non-template DNA, a permanent bubble was created. An initiation complex was shown to assemble after the binding of an RNA polymerase molecule to this bubble, in which an RNA oligonucleotide had previously been annealed with

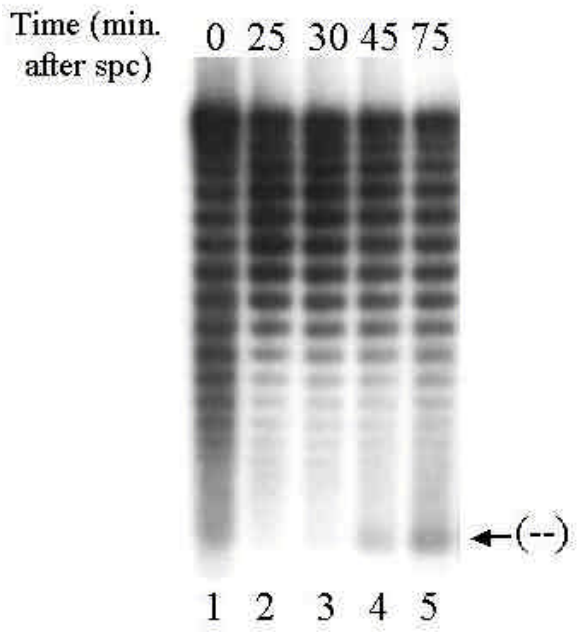

Figure 2. Time course of pBR322 hypersupercoiling in a *topA* null mutant in the presence of spectinomycin. DM800 cells *(deltatopA gyrB225*) carrying pBR322 were grown in LB medium with ampicillin at 37^0C to an $O.D._{600}$ of 0.5 at which time spectinomycin was added at 400 micrograms/ml. The cells were further incubated at 37^0C for 15 minutes before being transferred to 28^0C, a temperature that stimulates the accumulation of hypernegatively supercoiled plasmid DNA (42). At the indicated time, plasmid DNA was extracted and electrophoresed in the presence of 7.5 micrograms/ml of chloroquine as described (42). (--) indicates hypernegatively supercoiled DNA.

the template DNA strand. Proper RNA displacement from the RNA polymerase was shown to occur normally during transcription initiated in this way. However, the actively displaced nascent RNA was shown to reanneal with the template DNA strand within the permanent bubble. The presence of a permanently unwound DNA region behind a moving RNA polymerase can be considered as an optimal condition for R-loop initiation in the frame of this model.

Another interesting observation supporting the fact that nascent free RNA can be involved in R-loop formation, is the demonstration that RecA protein, involved in homologous recombination, can actively promote R-loop formation *in vitro*. The participation of RecA protein in R-loop formation was also suggested to take place in *rnhA* mutants (55). The involvement of RecA in hypernegative supercoiling and R-loop formation in *topA* null mutants is difficult to test since double *topA recA* is sick and seems to accumulate other mutations (Broccoli and Drolet, unpublished results). Therefore, our results on that matter do not allow us to reach a definitive conclusion.

4.3. Effects of translation inhibitors on hypernegative supercoiling and R-loop formation

Since transcription and translation are tightly coupled in bacteria, the presence of a ribosome sitting on the nascent RNA very close to the RNA polymerase should prevent the reannealing of this nascent RNA with the template strand behind the moving RNA polymerase. Obviously, under such conditions R-loop formation by the second mechanism will be inhibited, if it initiates within the translated portion of the RNA. Since ribosomes movement can reduce RNA polymerase pausing (56), one can presume that stalled RNA polymerases with extended RNA-DNA hybrids (the first mechanism) will also be displaced by ribosomes. In agreement with the inhibition of R-loop formation by translation, we found that the addition of translation inhibitors such as spectinomycin, chloramphenicol or kanamycin, stimulates hypernegative supercoiling in *topA* null mutants (32, 42). In some cases, when transcription is directed by a weak promoter, hypernegative supercoiling is found to be completely dependent on the presence of translation inhibitors (Broccoli and Drolet, unpublished results). Moreover, we found that hypernegatively supercoiled DNA is much more stable, as measured by inhibiting transcription with rifampicin, when translation inhibitors are added to the growth medium. This is predictable if ribosomes remove R-loops, because in this situation hypernegative supercoiling becomes unconstrained and will be rapidly relaxed by DNA gyrase and/or topoisomerase IV.

In figure 2, we show a time course of hypernegative supercoiling of pBR322 in a *topA* null mutant following the addition of spectinomycin. One can see that, at first, the plasmid is hypernegatively supercoiled via the twin-domain model (lane 1), because it disappears upon the addition of the translation inhibitor. After a while R-loop-dependent hypernegative supercoiling starts to accumulate (lane 4) and reaches its maximum steady-state level roughly one hour after spectinomycin addition (not shown). Two comments need to be made here. First, it can be seen that supercoiling related to the twin-domain model generates a heterogeneous distribution of topoisomers as initially observed (26), whereas upon the addition of the translation inhibitor, a more bimodal distribution of topoisomers is observed (compare lanes 1 and 5). This is explained by the fact that R-loop formation is not initiated every time the gene is transcribed. If not initiated, plasmids will remain supercoiled, as if no transcription has occurred, because with translation being inhibited, no hypersupercoiling related to the twin-domain can be generated by transcription. If the R-loop is initiated, plasmid supercoiling will eventually reach its maximum (hypernegative) because of the piling-up of RNA polymerases or the extension of the R-loop, depending on the mechanism by which R-loop formation occurs. In the presence of translation, every time the gene is transcribed supercoiling is generated, which explain the heterogeneous distribution of topoisomers. The second comment is related to the kinetics of hypernegative supercoiling of pBR322 in the presence of translation inhibitors. We found that hypernegative supercoiling in the presence of such inhibitors accumulates rapidly when the promoter and DNA gyrase activity are strong enough. According the first mechanism of R-loop formation, the piling-up of RNA polymerases is limited by the frequency of transcription initiation and therefore by the strength of the promoter. In the case of the second mechanism, R-loop extension,

responsible for hypernegative supercoiling, should be very rapid once it is initiated and will therefore not be limited by the strength of the promoter. A highly active DNA gyrase, and therefore a higher supercoiling level, will increase the probability of R-loop initiation.

Although the inhibition of R-loop formation by translation is easily predictable, the use of translation inhibitors might have revealed other important clues about the mechanism and turnover of R-loops. Indeed, we have made two observations that are not easily explainable by the effects of translation inhibitors on transcription-translation coupling. First, although inhibition of translation should favor R-loop formation, we found that the addition of translation inhibitors renders hypernegative supercoiling almost totally resistant to RNase H overproduction, despite the fact that RNase H activity is stable for hours after translation inhibition (Broccoli *et al.*, unpublished results). This is the case for pBR322deltatet5' for which hypernegative supercoiling is inhibited by RNase H overproduction (32) in the absence of translation inhibitors, but not in the presence of such inhibitors that also stimulate its hypernegative supercoiling (Broccoli *et al*., unpublished results). Second, we found that the major contribution for hypersupercoiling of this plasmid comes from transcription of a *tetA* gene bearing a deletion that removes the 5' coding region, including the ribosome binding site and the ATG start codon. Surprisingly, despite the fact that *tetA* is not translated, the addition of translation inhibitors strongly stimulates hypernegative supercoiling of this plasmid. Although several hypotheses can be provided to explain these two observations (for example see ref. 42), our recent results strongly suggest that (p)ppGpp, a small effector that regulates transcription initiation and elongation (57), is involved directly or indirectly in hypernegative supercoiling (Broccoli *et al.*, unpublished results) It is known that (p)ppGpp concentration drops to zero following treatments with translation inhibitors (58). Our results of experiments with triple *topA relA* and *spoT* null mutants (with no (p)ppGpp produced), which are very sick, support this hypothesis. Changes in (p)ppGpp levels may also explain our previous observation that hypernegative supercoiling preferentially occurs at low temperatures, since we found that (p)ppGpp levels drop considerably in *topA* null mutants relative to isogenic strains, upon transferring the cells from 37 to 28^{o}C (Rallu and Drolet, unpublished results). Interestingly, such a drop in (p)ppGpp level is not observed when the *topA* null mutant overproduces RNase H. We are currently performing experiments to better understand this intriguing link between (p)ppGpp, topoisomerase I and RNase H (see also section 5). However, changes in transcription velocity may also explain the temperature effect on hypernegative supercoiling as previously discussed (42).

5. HYPERSUPERCOILING AND R-LOOP IN GENE EXPRESSION

When we think about RNase H in gene expression, one of the first things that comes into our mind is the degradation of RNA and the concomitant inhibition of protein synthesis, followed by growth inhibition. However, in the case of *topA* null mutants exactly the reverse is observed. Therefore, R-loops are inhibitory most likely because they inhibit transcription elongation by constituting roadblocks. In such cases, RNase H activity should increase the yield of full length RNA synthesis. This is precisely what is observed both *in vitro* and *in vivo*. Indeed, we have repeatedly observed the stimulation of RNA synthesis by RNase H, when negatively supercoiled templates are transcribed with T7 RNA polymerase (36, 39 and figure 3). This effect is enhanced by the addition of DNA gyrase in the reaction (Masse and Drolet, unpublished results), as predicted since R-loop formation is promoted by negative supercoiling. The effect of RNase H is also enhanced by a high ratio of RNA polymerases to template, which favors the hypothesis that R-loops constitute roadblocks for RNA polymerases. The same will be true *in vivo*: heavily transcribed genes will be those that are mostly affected by R-loop formation and therefore greatly stimulated by RNase H activity. The stimulation by RNase H of full length RNA synthesis by T7 RNA polymerase on a supercoiled template has also been observed by another group (51). They concluded that RNase H was either allowing the stalled RNA polymerase to resume transcription, or was stimulating the dissociation of the stalled polymerase to clear the passage for other RNA polymerases. It is also worth mentioning that under certain circumstances extended RNA-DNA hybrids are shown to displace RNA polymerases (59-60). Under such conditions, RNase H activity should not have a significant effect on transcription. We have also found that RNase H overproduction stimulates rRNA synthesis (61; Hraiky *et al*., unpublished results and see below) and *crp* synthesis (Baaklini and Drolet and see below) in *topA* null mutants.

5.1. R-loop formation and rRNA synthesis

The study of R-loop formation during rRNA synthesis was initiated because, first of all, rRNAs are not translated and therefore not bound by ribosomes. Second, *topA* null mutants and *rnhA* mutants are both particularly sensitive to nutritional shift-ups, conditions that elicit a very high rate of rRNA synthesis (see table 1 for a list of shared phenotypes between *topA* and *rnhA* mutants). As expected, R-loop formation was shown to occur in *topA* null mutants when a portion of the *rrnB* operon, one of the seven operons encoding rRNAs in *E .coli*, was transcribed on a plasmid *in vitro* in the absence of topoisomerase I (36, 39) and in *topA* null mutants *in vivo* (39). Interestingly, we found that transcription of this DNA fragment preceded by a wild-type *boxA*, but not a mutated one, was causing plasmid instability when RNase H was not overproduced in the *topA* null mutant (39). Plasmid instability was possibly due to the presence of an R-loop or a stalled polymerase, that was impeding the progression of a replication fork. *boxA* is the minimal sequence that acts in *cis* at the RNA level to build an antitermination system for rRNA synthesis. Such a system, that also increases the velocity of the RNA polymerase (62), is required to prevent *rho*-dependent premature termination of rRNA synthesis (63). This antitermination system plays the same role for the rRNAs as the ribosomes play for the translated mRNAs, in preventing premature termination. In order to test the involvement of the *boxA* sequence in R-loop formation and inhibition of transcription elongation, we constructed *lacZ*

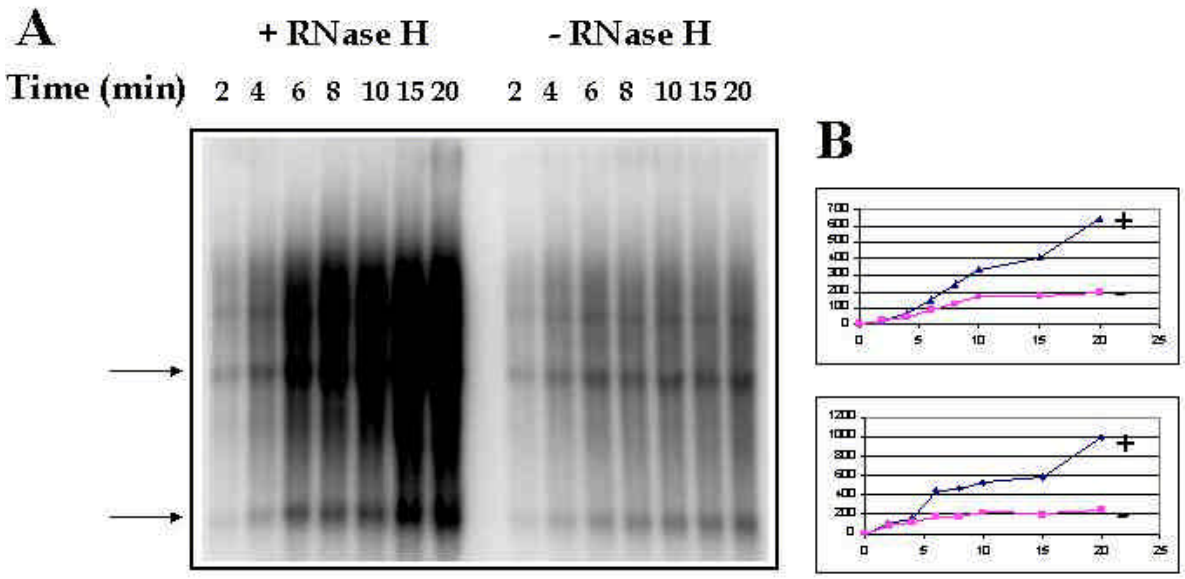

Figure 3. Kinetics of *in vitro* transcription by T7 RNA polymerase on a supercoiled template in the presence or absence of RNase H. *In vitro* transcription reactions with T7 RNA polymerase in the presence or absence of RNase H were performed as described previously (39), except that [^{32}P]-UTP was added to label the RNA. Negatively supercoiled pJP461 (39) was used in the transcription reactions. In this plasmid, the 567 bp *Hin*dIII fragment from the *rrnB* operon is transcribed in its physiological orientation by T7 RNA polymerase. At the indicated time, an aliquot was recovered and the RNA was extracted and electrophoresed in a denaturing agarose gel. **A**. Autoradiogram of the gel after electrophoresis. The arrows on the left indicate the bands that have been quantified by densitometry. **B**. Densitometry of the bands pointed by an arrow in A. The top and the bottom panels respectively correspond to the top and the bottom bands. + and – means that RNase H was respectively present and absent during transcription.

fusions. The *boxA* sequence was cloned downstream of the IPTG-inducible P*trc* promoter and upstream of the *lacZ* gene. We found that *lacZ* expression as assayed by measuring beta-galactosidase activity is barely detectable in the *topA* null mutant that does not overproduce RNase H (Demers *et al*., unpublished results). We have shown that initiation at P*trc* takes place normally whether RNase H is overproduced or not and that the *topA* null mutant not overproducing RNase H accumulates new small RNA species (Demers *et al*., unpublished results). This results suggest that R-loops are inhibiting transcription elongation of the *lacZ* gene in this construct. More experiments are being performed to understand the effect of the *boxA* sequence.

Following the observation that R-loop formation could occur during rRNA synthesis, we performed a series of experiments that demonstrated a significant reduction of the rRNA synthesis rate, at the level of transcription elongation, in *topA* null mutants (61). Since rRNA synthesis was reestablished to a normal level upon RNase H overproduction, we concluded that stalled RNA polymerases with extended RNA-DNA hybrids, were acting as roadblocks for the next RNA polymerases (61). In agreement with the existence of stalled RNA polymerases, we detected the presence of very stable RNA species carrying the rRNA leader region, that rapidly disappeared when RNase H was overproduced. Interestingly, one of the stable RNA products was localized within a region where R-loop formation was previously shown to occur (39 and see above). Other stable RNA products much closer to the 5' end of the rRNA leader were also detected not only in *topA* null mutants, but also in *rnhA* and double *deltarelA deltaspot* ((p)ppGpp^0) mutants (Hraiky *et al*., unpublished results). The fact that the accumulation of these products is not specific to *topA* null mutants may indicate that it is related to global supercoiling and therefore not linked to the major function of topoisomerase I. At the same time, these results reveal that R-loop formation may occur at physiological global supercoiling. For the second time, the results of these experiments reveal an intriguing relationship between (p)ppGpp, topoisomerase I and RNase H (see also section 4.3). The stable RNA species with 3' ends downstream of the rRNA leader region are only detected in *topA* null mutants (Hraiky *et al*., unpublished results). Therefore, in this case, R-loop formation is probably related to local supercoiling which is linked to the specific and major function of topoisomerase I.

5.2. R-loop formation and mRNA synthesis

In principle, there are no reasons why mRNA synthesis should not be susceptible to and therefore inhibited by R-loop formation, despite the fact that the coupling of transcription with translation can minimize their effects. Since our results have revealed that the major *cis*-acting determinant of R-loop formation is the level of negative supercoiling and not the nucleotide sequence, it can probably take place at many loci. Moreover, in the case where extended RNA-DNA hybrids destabilize the RNA

Table 1. Shared phenotypes between *topA* and *rnhA* null mutants[1]

A. Growth Phenotypes	References
Sensitivity to nutritional shift-ups (stationary phase to fresh medium and poor to rich media)	15
Sensitivity to growth on MacConkey plates	15
Sensitivity to low temperatures	42, 65
Growth inhibition in combination with nusB mutations[2]	39 and Drolet, unpublished results
Growth inhibition by a plasmid (pNO1302) carrying a portion of the rrnB operon[3]	39 and Drolet, unpublished results
Growth improvement by a gyrB(Ts) allele	15
B. Gene expression phenotypes	
Accumulation of very stable short RNAs from the leader region of rrn operons[4]	Hraiky and Drolet, unpublished results
Drop in crp expression level at low temperatures	Baaklini and Drolet, unpublished results
Sensitivity to heat-shock	13, Fortin and Drolet, unpublished results

[1] In all cases the phenotypes are more severe for the *topA* null mutants and overproduction of RNase H in these *topA* mutants alleviates the phenotypes. [2]The growth of a double *nusB5 topA* null mutant is barely detectable despite the presence of a *gyrB* compensatory allele. It is not possible to introduce a *nusB* null allele into a *topA* null mutant.[3] The plasmid pNO1302 is a pBR322 derivative in which a portion of the *rrnB* operon is transcribed from its own promoters. We also found that overproducing RNase H improves the growth of a wild-type strain carrying this plasmid without changing the plasmid copy number. [4] This phenotype is also shared with a (p)ppGpp^0 *(deltarelA deltaspoT*) strain and is preferentially expressed at low temperatures.

polymerase and therefore cause premature termination, transcription inhibition will not be revealed by RNase H overproduction. In other words, even if RNase H overproduction has no relieving effects on the expression of a gene, R-loop formation may still be responsible for transcription inhibition of this gene.

In agreement with the assumption that R-loop formation may inhibit the expression of genes encoding for mRNAs, is the finding that *crp* expression is reduced in a *topA* null mutant not overproducing RNase H (Baaklini and Drolet, unpublished results). Also supporting the effect of R-loops on mRNAs synthesis is the finding that RNase H overproduction improves the survival of *topA* null mutants to heat-shock and oxidative stress (Yuk-Ching Tse-Dinh, personal communication).

The currently available data allow us to draw models for R-loop formation and its effect on transcription elongation (figure 1). Whether R-loop formation is initiated from the transcription bubble or by the reannealing of the nascent RNA with the template strand behind the RNA polymerase, R-loops inhibit transcription by acting as roadblocks for RNA polymerases.

6. PERSPECTIVES

So far, discussions of R-loops in transcription have been restricted to inhibitory effects and therefore not to the regulation of gene expression. Indeed, the fact that the *topA* gene possesses promoters controlled by different sigma factors (11-12), indicates the need to express topoisomerase I whether bacterial cells are under unrestricted growth conditions or under stress. In this context, topoisomerase I could almost be considered as a housekeeping transcription factor. On the other hand, the possible link between RNase H, (p)ppGpp and global supercoiling, may reveal a regulatory role of R-loop on gene expression. It is worth mentioning the results of one study that have revealed a correlation between the pausing time of an RNA polymerase and the template negative supercoiling level, during *in vitro* transcription of the *rrnB* leader region (64). Whether R-loop formation is involved in RNA polymerase pausing remains to be seen. The outcome of these studies on R-loop formation in gene expression should contribute to our understanding of the mechanism of transcription on negatively supercoiled templates.

Another important aspect of the physiology of R-loops that has not been discussed here, is their possible involvement in other DNA functions. The well-described phenomenon of constitutive stable DNA replication (cSDR) that occurs in *rnhA* mutants (65) that allows the bacterial cell to survive without their normal origin of replication (*oriC*), is a good example of the involvement of R-loops in replication initiation. Interestingly, cSDR is inhibited by the stringent response that increases the cellular concentration of (p)ppGpp and is stimulated by the addition of chloramphenicol that brings the cellular (p)ppGpp concentration to nearly zero. In this context, it is interesting to mention the results of a recent study that have strongly implicated (p)ppGpp in the removal of stalled RNA polymerases at lesions in the DNA (66). This (p)ppGpp effect allows the cells to better survive from DNA damages by apparently improving the restart of replication forks. We are currently studying the *boxA* and R-loop-dependent plasmid instability in *topA* null mutants, to verify if stalled RNA polymerases via R-loops are blocking the passage of replication forks. Such a possibility has been suggested to explain the arrest of a replication fork by a stalled RNA polymerase at d(G)n.d(C)n repeats (67). The involvement of R-loop formation in genome instability has also been evoked among other hypotheses to explain transcription-mediated recombination in yeast (68).

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Send correspondence to: Dr Marc Drolet, Departement de microbiologie et immunologie, Universite de Montreal, C.P. 6128, Succ. Centre-ville, Montreal, P.Quebec, Canada, H3C 3J7. Tel : 514-343-5796, Fax : 514-343-5701, E-mail : marc.drolet@umontreal.ca