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Rôle de la topoisomérase I durant la transcription chez *Escherichia coli*

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1 Sommaire

Les ARN produits chez la bactérie *Escherichia coli* sont généralement amenés vers deux voies distinctes. Le plus souvent, l'ARN naissant se dissocie de la matrice, durant sa synthèse, afin d'être fonctionnel. Celui-ci peut alors servir de matrice pour les ribosomes qui le traduisent en polypeptides, ou alors il peut servir de matériau de structure dans la composition des ribosomes. Le second type de transcrit trouve son utilité lorsque l'ARN demeure attaché à la matrice ADN afin d'amorcer la réplication. Ce type d'ARN est très court et il est immédiatement utilisé comme amorce par la polymérase ADN de la cellule. Récemment, il a été démontré que les hybrides ARN-ADN peuvent aussi être impliqués dans la recombinaison. Plusieurs travaux menés sur la formation de ces structures hybrides indiquent que celle-ci dépend généralement du surenroulement de l'ADN et de certains paramètres de transcription.

La formation d'hybrides ARN-ADN durant la transcription démontre bien que la compréhension des mécanismes de transcription serait évidemment incomplète sans y inclure la topologie de l'ADN. L'étude de la transcription révèle un mécanisme dynamique très complexe, finement régulé à plusieurs niveaux, tant à l'initiation, l'élongation, que la terminaison. La topologie de l'ADN, ainsi que les topoisomérases ADN ont été caractérisées comme des modulateurs importants dans toutes les étapes de l'expression génique, allant de la reconnaissance spécifique des promoteurs jusqu'au déplacement de l'ARN naissant ou de son attachement à la matrice ADN. De plus, la transcription est l'évènement génétique le plus fréquent dans une cellule.

Selon le travail présenté ici, un des rôles essentiels de la topoisomérase I semble être l'inhibition de la formation d'hybrides ARN-ADN durant la transcription chez *Escherichia coli*. Ceux-ci semblent survenir principalement au niveau des opérons ribosomaux. Le couplage entre la traduction et la transcription, chez les ARNm, aide à prévenir la formation d'hybrides ARN-ADN. La croissance à basse température favorise l'appariement de l'ARN à l'ADN. La suite des travaux décrits propose que le rôle essentiel de la topoisomérase I chez *Escherichia coli* n'est pas la relaxation du surenroulement global de l'ADN, mais plutôt l'élimination du surenroulement local

généré durant la transcription. Ce surenroulement semble responsable de l'initiation de la formation de structures hybrides ARN-ADN, alors que la gyrase provoquerait leur élongation. Ces observations permettent de suggérer de nouveaux rôles pour la topoisomérase I et la gyrase chez les bactéries.

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4   Listes des abréviations

aa :	acide aminé
ADN :	acide désoxyribonucléique
ADNmt :	ADN mitochondrial
ARN :	acide ribonucléique
ARNm :	ARN messager
ARNr :	ARN ribosomal
ARNt :	ARN de transfert
CsCl :	chlorure de césium
E :	noyau de la polymérase ARN
E. coli :	*Escherichia coli*
σ :	facteur sigma
EDTA :	acide éthylènediamine tétraacétique disodique
nt :	nucléotide
ppGpp :	guanosine tétraphosphate
pb :	paire de base
SDS :	dodécyl sulfate de sodium
ssb :	« single strand DNA binding protein »
kb :	kilobases
ATP :	adénosine triphosphate
ADP :	adénosine diphosphate
CSB :	« conserved sequence block »
RNase :	ribonucléase
LSP :	« light-strand promoter »
HSP :	« heavy-strand promoter »
Tn :	transposon

À Stéphanie

6   Introduction

6.1   Polymérases ARN

6.1.1   Le complexe de transcription

Les différentes étapes du cycle transcriptionnel, effectué par la polymérase ARN sont : la liaison au promoteur, la séparation des deux brins ADN, l'initiation de la synthèse de l'ARN, l'élongation et la terminaison de la transcription. Chacune de ces étapes spécifiques est sujette à une régulation précise afin de moduler l'expression du gène transcrit. La polymérase ARN de *Escherichia coli* (*E. coli*) peut transcrire de manière efficace (processivité élevée) jusqu'à 10^4 nucléotides à une vitesse pouvant atteindre 100 nt/sec (Landick, 1997).

La polymérase ARN active existe sous au moins deux formes différentes. La première formes (E) constitue le noyau de l'enzyme et est composée des sous-unités α, β et β' dans un ratio respectif de 2:1:1. La seconde forme, l'holoenzyme, consiste en l'association du noyau avec le facteur σ (Eσ). Ce dernier n'est nécessaire que durant l'initiation de la transcription et il se dissocie rapidement après cette étape. Plusieurs types de facteur σ existent afin d'initier la transcription à des promoteurs spécifiques. Le principal facteur σ chez *E. coli* est σ^{70}.

6.1.2   Structure moléculaire

Afin de comprendre les interactions protéine-ADN importantes pour le fonctionnement de la polymérase ARN, le concept de pince glissante (« sliding clamp »), analogue au modèle de la polymérase ADN (Kuriyan and O'Donnell, 1993), fut proposé (Nudler *et al.*, 1996). La comparaison entre la structure d'une polymérase ARN au moment de l'initation de la transcription (holoenzyme) et durant l'élongation (sans facteur σ) suggère la formation d'un anneau entourant l'ADN. Celui-ci se forme lors d'une transition de structure de la protéine lorsqu'elle se dégage du promoteur (« promoter clearance »; Polyakov *et al.*, 1995). Cependant, contrairement à la

polymérase ADN, la polymérase ARN doit résoudre le problème éventuel de séparer le produit de transcription attaché au brin matrice ADN.

Selon les données récentes d'empreintes et de cristallographie, le complexe de transcription de *Escherichia coli* se divise en trois régions contiguës et distinctes par leurs structures et leurs fonctions. Chacune de ces régions se lie à un site particulier, soit dans l'ADN ou l'ARN. Ces régions sont le site d'attachement à l'ADN double-brin, le site d'attachement à l'hybride ARN-ADN et le site d'attachement à l'ARN naissant simple-brin. Le site d'attachement à l'ADN double-brin s'étend sur les 9 paires de bases (pb) situées juste en aval de l'ouverture des brins formant la bulle de transcription (Nudler *et al.*, 1996). Le second site, interagissant avec l'hybride, à été localisé entre la protéine et les six paires de bases situés immédiatement en amont du site catalytique. Les expériences de marquages (« footprinting ») et de « cross-linking » entre l'ARN et l'ADN suggèrent la présence d'un hybride de 8-10 pb dans la région 3' de l'ARN nouvellement transcrit (Lee and Landick, 1992; Zaychikov *et al.*, 1995; Nudler *et al.*, 1997). Cependant, la longueur de l'hybride fait l'objet d'un débat encore actif et varie considérablement selon les approches expérimentales (voir plus loin). Le troisième et dernier site d'interaction, celui de l'ARN simple-brin, fut proposé par Kumar et Krakow (1975) à l'aide d'expériences de protection de l'ARN par le complexe de transcription. L'existence de ce site fut également supporté par une démonstration récente de l'attachement équimolaire de la polymérase ARN à l'ARN, formant ainsi un complexe binaire (Altmann *et al.*, 1994). Ce site d'interaction se localise sur une distance de 9 nucléotides immédiatement après l'hybride ARN-ADN (Nudler *et al.*, 1998). Les sous-unités α, β et β' de la polymérase démontrent un contact avec l'ARN mais ces interactions ne semblent pas cependant contribuer à maintenir la stabilité du complexe (Komissarova and Kashlev, 1998). Cette région semble plutôt fortement impliquée dans la modulation des sites de pause et de terminaison (Liu *et al.*, 1996).

6.1.3   Modèles du complexe d'élongation

Plusieurs modèles tentant d'expliquer le mécanisme d'élongation de l'ARN ont été proposés. Le premier modèle, appelé classique, suggère qu'un hybride ARN-ADN

d'environ 12 pb permet d'empêcher la déstabilisation du complexe de transcription (Gamper and Hearst, 1982; Yager and von Hippel, 1991). Dans ce modèle, la polymérase ARN progresse de manière monotone en synchronisme avec l'ajout des nucléotides. La terminaison se produit par la déstabilisation de l'hybride résultant des interactions faibles rU-dA, de concert avec la formation d'une structure tige-boucle que l'on retrouve chez les terminateurs de transcription.

Un second modèle, nommé « révisionniste », sous-entend que deux sites seulement dans la protéine, l'un devant et l'autre derrière, garderaient un contact solide avec l'ADN et maintiendraient la stabilité du complexe. Selon les empreintes d'ARN, l'hybride interne ne mesurerait que 2-3 pb, et ne pourrait contribuer au maintient de la polymérase sur la matrice (Chamberlin, 1995). Dans ce modèle, la polymérase fonctionne selon un cycle « contraction/expansion » partagé en deux temps. Dans le premier temps (contraction), l'enzyme est statique et un court segment d'ARN est synthétisé (2 pb ou plus). Ensuite, la polymérase se déplace de quelques nucléotides vers l'avant (expansion) afin de recommencer le cycle. Les évidences supportant ce modèle proviennent d'expériences de « footprints » de polymérases ARN arrêtées indiquant des dimensions variables (Krummel and Chamberlin, 1992; Nudler et al., 1994). Cependant, de récentes données ont forcé une nouvelle interprétation de ces résultats. En effet, il fut démontré que dans certaines conditions, la polymérase ARN pouvait glisser librement vers l'arrière sur la matrice ADN et l'ARN, entraînant avec elle la bulle de transcription. Conséquemment, l'extrémité 3' de l'ARN serait éloignée du site actif de l'enzyme. De cette manière, le « footprint » pourrait paraître plus petit ou plus grand selon l'importance du glissement (Komissarova and Kashlev, 1997).

Il existe actuellement un troisième modèle de la structure du complexe de transcription. Celui-ci est peut-être le plus proche de la réalité puisqu'il explique les résultats contradictoires des deux modèles précédents. Ce modèle découle de l'utilisation d'oligonucléotides ADN et ARN synthétiques en plus de la polymérase ARN, ce qui forme un complexe de transcription ternaire. La technique des oligonucléotides synthétiques a permis de développer l'hypothèse que l'hybride

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ARN-ADN est primordial à la synthèse processive d'ARN et que celui-ci devait mesurer au moins 9 nt pour maintenir la stabilité du complexe (Sidorenkov *et al.*, 1998).

La stabilité latérale (c'est-à-dire la résistance au glissement) du complexe de transcription dépendrait de la force de l'hybride ARN-ADN interne (Nudler *et al.*, 1997). Un « footprint » de dimension variable pourrait découler du recul de la polymérase ARN lorsqu'elle est arrêtée à un endroit où l'hybride est instable, et ce, afin d'en reformer un de plus grande stabilité (Landick, 1997). Dans le cas de l'incorporation d'un mauvais rNTP par exemple, l'instabilité de l'hybride rendrait le complexe incompétent à l'élongation (Erie *et al.*, 1993). La polymérase ARN de *E. coli* possède une faculté intrinsèque de clivage de l'ARN (Orlova *et al.*, 1995) qui serait d'ailleurs stimulée par les facteurs de clivage du transcrit, GreA (Erie *et al.*, 1993) et GreB (Borukhov *et al.*, 1993). L'ARN clivé se dissocie ensuite du transcrit et la polymérase peut alors poursuivre l'élongation à partir de la nouvelle extrémité 3' du transcrit, ce qui augmente la fidélité de ce dernier.

6.1.4 Transcription et surenroulement

Les acides nucléiques ont des propriétés physico-chimiques variables qui influencent leur qualité en tant que substrats d'enzymes. Déjà en 1965, Hayashi notait que les formes réplicatives I et II de l'ADN du phage ϕX174 démontraient une activité de transcription différente l'une de l'autre. Les résultats obtenus suggéraient que la forme réplicative I de l'ADN (aujourd'hui on sait qu'elle est surenroulée) était beaucoup plus active que la forme II (ADN relaxé). Peu après, il fut aussi démontré que la forme surenroulée négativement du chromosome de SV40 était un meilleur substrat pour la polymérase ARN d'*E. coli* que le même chromosome sur lequel était présent une cassure simple-brin, donc relaxée (Westphal, 1970). D'autres expériences reliées au surenroulement de l'ADN démontrèrent que l'attachement d'un ligand à l'ADN se trouve favorisé par la présence de supertours négatifs (Vinograd *et al.*, 1968; Bauer and Vinograd, 1968, 1970; Davidson, 1972). Ainsi, tous ces travaux indiquaient que l'interaction enzyme-ADN pouvait varier en fonction de la densité de surenroulement de l'ADN.

6.1.5 Formation d'un hybride ARN-ADN durant la transcription

La polymérase ARN de *E. coli* est une protéine qui déroule l'ADN en s'y attachant (Saucier and Wang, 1972). Chez ce type de protéine, les différences structurales entre l'ADN surenroulé et l'ADN relaxé feront varier considérablement la constante d'attachement (Davidson, 1972). Les études subséquentes indiquèrent que l'activité de transcription de la polymérase ARN croît rapidement en fonction de la densité de surenroulement négatif de l'ADN (Botchan *et al.*, 1973; Wang, 1974). Donc, selon ces résultats, la polymérase ARN démontre d'avantage d'affinité et d'activité avec l'ADN surenroulé qu'avec l'ADN relaxé. De plus, à l'aide de matrices ADN de différentes densités de surenroulement, Wang (1974) observa que l'ARN nouvellement transcrit *in vitro* s'hybridait de manière stable avec le brin ADN homologue, mais seulement à partir d'une densité de surenroulement négative relativement élevée (dans cette étude : -0.045). Le surenroulement négatif de l'ADN tend à séparer l'un de l'autre les brins homologues de la double hélice. L'interprétation la plus simple de ces données était que sur une matrice surenroulée négativement, l'ARN nouvellement transcrit peut facilement s'apparier avec le brin ADN matrice, formant ainsi un hybride ARN-ADN (R-loop). Sur une matrice ADN relaxée, on observe plutôt un resserrement dans l'appariement des deux brins

Peu de temps après, une seconde étude de transcription *in vitro* sur des matrices surenroulée et relaxée fut publiée (Richardson, 1975). Dans ce cas, cependant, les réactions de transcriptions furent arrêtées avec différents inhibiteurs pouvant dénaturer ou non les protéines. Ces produits étaient soit de l'EDTA, du SDS, du CsCl, de l'urée ou du phénol. Lorsque la réaction fut terminée à l'aide d'un agent non dénaturant, l'EDTA, l'une ou l'autre des matrices (relaxée ou surenroulée) ne portait pas de fragments d'ARN hyridés plus long que 20 nucléotides. Ceci démontrait que l'ARN nouvellement transcrit était déplacé de la matrice au fur et à mesure qu'il était synthétisé. Cependant, l'arrêt de la réaction par l'ajout des produits dénaturants les protéines (le SDS, le CsCl, l'urée ou le phénol) induisait une importante formation d'hybrides ARN-ADN, et ceci, uniquement sur la matrice surenroulée. Dans ces conditions, on pouvait observer que 60% de l'ARN synthétisé s'attachait à la matrice,

et ce, sur une longueur pouvant atteindre jusqu'à 600 nucléotides. À la lumière de ces résultats, il devenait probable que les résultats de Wang (1974) étaient artéfactuels, puisque celui-ci utilisait, pour arrêter les réactions, des techniques qui dénaturaient les protéines. Une interprétation importante de ces nouvelles données était que l'ARN naissant, provenant d'une matrice relaxée ou surenroulée négativement, était normalement activement déplacé durant la transcription. Finalement, comme l'attachement de l'ARN à la matrice ne s'observait que lorsque la polymérase ARN était dénaturée, ceci indiquait que la polymérase ARN de *E. coli* possède une fonction intrinsèque favorisant le déplacement de l'ARN nouvellement transcrit.

Les expériences de Wang (1974) et de Richardson (1975) démontraient bien la relation délicate et complexe qui existe, à l'intérieur du complexe ternaire de transcription, entre le substrat (ADN), le produit (ARN) et l'enzyme (polymérase ARN).

6.2 Surenroulement de l'ADN

6.2.1 Fonctions et activités des topoisomérases

Certaines fonctions génomiques peuvent générer des tensions de surenroulement extrêmement élevées dans la molécule d'ADN. La réplication est l'un des meilleurs exemples puisque la polymérase ADN, qui avance à plus de 800 nucléotides/secondes, génère des supertours positifs. Ces derniers doivent être rapidement éliminés afin de ne pas entraver la progression de la fourche de réplication. Dans ce cas, la coupure de un des deux brins ADN permettra de libérer l'accumulation de surenroulement. Un autre problème survient à la fin de la réplication; l'enchaînement des deux molécules filles d'ADN l'une à l'autre. Pour les séparer (décaténation), les deux brins ADN de l'une des deux molécules doivent être coupés. Afin de résoudre ces deux problèmes, la cellule utilisera respectivement l'activité des topoisomérases de type I, qui coupent un brin d'ADN à la fois, ou celle de type II, qui coupent deux brins d'ADN à la fois. L'activité complète des topoisomérases, qu'elle soit de type I ou II, requiert, dans l'ordre, la coupure d'un brin

(ou des deux brins), puis le passage de l'autre brin (ou de l'ADN double-brin) au travers de la coupure et finalement la religation de la (ou des) cassure(s).

Quatre topoisomérases ADN ont été découvertes chez *E. coli*. Celles-ci sont la topoisomérase I (Wang, 1971), la gyrase (topoisomérase II; Gellert *et al.*, 1976), la topoisomérase III (Dean *et al.*, 1983) et la topoisomérase IV (Kato *et al.*, 1990). Les topoisomérases I et III sont de type I (coupent un brin ADN), alors que la gyrase et la topoisomérase IV sont de type II (coupent deux brins ADN). De ces quatre enzymes, il n'y a que la gyrase qui puisse introduire du surenroulement négatif dans l'ADN. Elle peut aussi relaxer l'ADN positif et, selon les conditions expérimentales (voir section 6.2.5), relaxer l'ADN surenroulé négativement, tout comme les topoisomérases I et III. La topoisomérase IV de *E. coli*, ainsi que les topoisomérases I et II de types eucaryotes, peuvent relaxer les supertours positifs et négatifs (Wang, 1996). Ces topoisomérases sont impliquées dans différentes fonctions de l'ADN, dont la transcription, qui sera discutée de manière plus approfondie.

6.2.2 Modèle du « twin-supercoiled-domain »

Tout comme l'élongation du complexe de réplication, la synthèse d'ARN par la polymérase ARN soulève plusieurs problèmes inhérents à la structure hélicoïdale de l'ADN. Il est en effet difficile d'imaginer que la polymérase ARN, ainsi que l'ARN nouvellement transcrit couvert de ribosomes, puissent tourner autour de l'ADN lors de la transcription. Une autre possibilité est que l'ADN tourne autour de son axe durant l'élongation du complexe de transcription. Si la dernière hypothèse est favorisée, il faut tenir compte qu'à chaque tour de l'hélice Watson-Crick traversé par la polymérase (environ 10 pb), un supertour positif sera créé devant et un négatif derrière.

La résolution des topoisomères de pBR322 extraits d'une souche sauvage donne une distribution normale, c'est-à-dire de type Gaussienne, dans laquelle le surenroulement de l'ADN est négatif. Toutefois, l'analyse topologique du même plasmide, extrait de souches dont la gyrase est inhibée par des drogues, démontre la présence de topoisomères surenroulés positivement (Lockshon and Morris, 1983).

Lorsqu'il est extrait d'un mutant *topA*, la distribution du pBR322 devient alors très hétérogène, et de plus, il apparaît un type particulier de topoisomères démontrant une densité de surenroulement hautement négatif (2 à 3 fois supérieure à celle mesurée chez une souche *topA+*; Pruss, 1985), aussi appelés plasmides hypernégativement surenroulés. Dans une autre expérience, on nota également que la distribution aberrante des topoisomères provenant d'un mutant *topA* semblait liée à la transcription du gène *tetA*, situé sur le plasmide pBR322. En effet, l'inactivation de ce gène est suffisante pour que les plasmides retrouvent une distribution topologique normale (Pruss and Drlica, 1986).

Les résultats de ces multiples expériences ont été rassemblés et interprétés afin de définir un modèle général, soit le « twin-supercoiled-domain » (Liu and Wang, 1987). Ce modèle soutient que, durant l'élongation de la transcription par la polymérase ARN, il se forme en amont et en aval de l'enzyme, des domaines de surenroulement négatifs et positifs respectivement. Ces domaines de surenroulement seraient normalement éliminés par l'activité de l'enzyme topoisomérase I, qui relaxe l'ADN surenroulé négativement, et la gyrase, qui relaxe le surenroulement positif. Ainsi, lorsque le complexe de transcription se déplace sur la matrice ADN, les topoisomérases créent un véritable pivot permettant ainsi à la polymérase ARN de progresser dans une zone « neutre » de surenroulement.

Selon ce modèle, le déplacement d'une protéine qui déroule l'ADN, soit durant la réplication ou la transcription, génèrera à cet endroit précis un surenroulement transitoire appelé surenroulement local. Ce dernier est différent du surenroulement global qui demeure immuable et représente la densité de surenroulement moyen d'une molécule entière d'ADN. Sur une matrice circulaire, les supertours positifs et négatifs générés durant l'élongation de la transcription vont éventuellement diffuser dans le cercle l'un vers l'autre et s'annuler lors de leur rencontre (Fig. 1A). Cependant, dans la cellule, plusieurs facteurs peuvent créer une accumulation des tensions dans l'ADN durant l'élongation de la transcription. Par exemple, la présence de ribosomes sur le transcrit augmentera le poids du complexe de transcription, et ainsi, amplifiera la tension générée dans la matrice. D'autres situations telles la présence de protéines sur

la matrice (histones, polymérase ADN et autre polymérase ARN), ou l'attachement de l'ADN à la membrane empêcheront la diffusion des supertours générés durant l'élongation par la polymérase ARN.

Toujours selon le modèle du « twin-supercoiled-domain », la présence de deux ou plusieurs polymérases ARN en mouvement auront plus ou moins d'impact dépendemment de leur direction relative. Deux complexes de transcriptions se déplaçant dans la même direction n'auront pas tellement plus d'impact qu'un seul. La région ADN entre les deux polymérases sera relaxée dû à l'annulation des supertours négatifs et positifs alors qu'on retrouvera un domaine de surenroulement positif devant et négatif derrière (Fig. 1B). Cependant, dans une situation où les deux complexes sont divergents, 2 domaines topologiques apparaîtront. Le segment ADN situé entre les polymérases ARN sera surenroulé négativement tandis que les régions flanquantes seront positives.
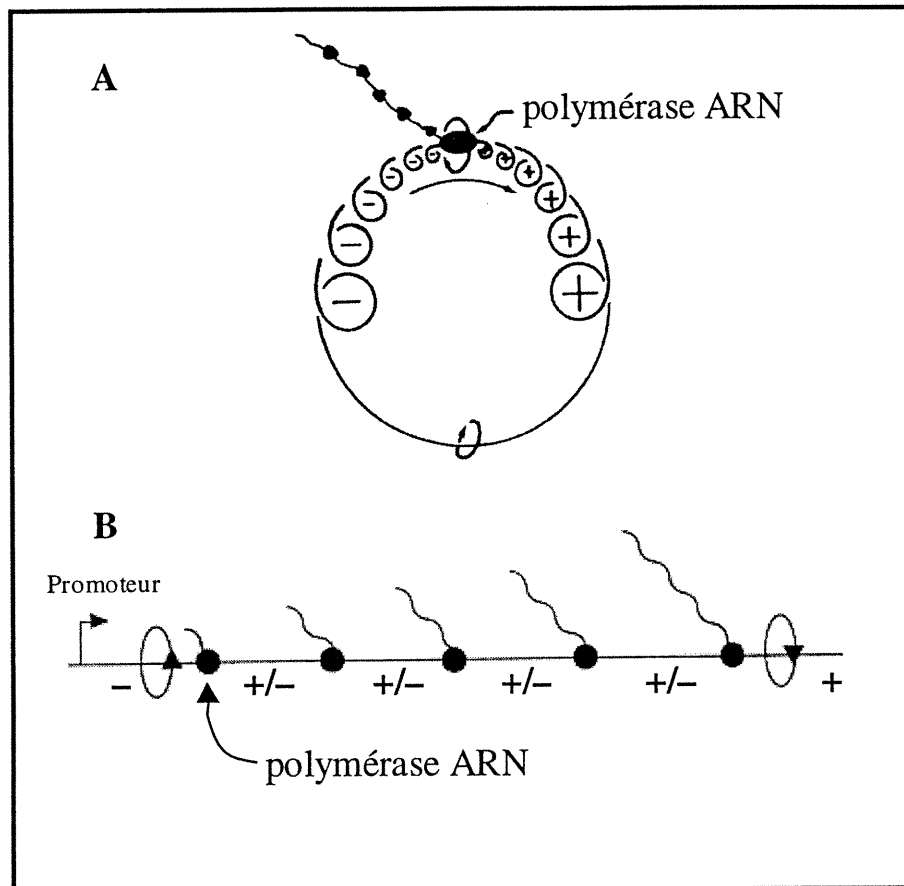
Figure 1 : Modèle du « twin-supercoiled-domain ». A. Le mouvement de la polymérase ARN créer, devant et derrière, du surenroulement positif et négatif respectivement. B. Plusieurs complexes de transcription sur la molécule ADN vont générer des domaines de surenroulement qui s'annuleront entre les régions transcrites. Tiré de Tsao *et al.*, (1989) et de Drolet *et al.*, (1994b).

6.2.3 La transcription du gène *tet*

Le modèle du « twin-supercoiled-domain » permettait aussi d'expliquer les résultats obtenus avec le pBR322 provenant des souches mutantes pour la topoisomérase I. Chez les procaryotes, puisque la transcription et la traduction sont couplées, on aura, dans le cas d'une protéine membranaire (ex. : TetA), ancrage de la queue N-terminale du polypeptide dans la membrane cellulaire alors que la transcription se déroule encore. Dans cette situation, la rotation de la polymérase est complètement bloquée. On peut observer le même principe lorsque la polymérase transcrit une région de l'ADN alors qu'elle interagit simultanément avec une seconde région de l'ADN. Ceci formera, entre les deux points de contact de la polymérase avec la matrice, un domaine topologique fermé où le surenroulement pourra s'accumuler. Un autre exemple possible, est lorsqu'un facteur quelconque, qui empêche les supertours de diffuser, s'associe avec l'ADN en amont ou en aval de la polymérase. Finalement, l'appariement de l'ARN naissant avec son ADN homologue (R-loop) ou avec un second ARN naissant homologue transcrit de manière divergente empêchera aussi la rotation de la polymérase.

Suite à sa proposition, le modèle du « twin-supercoiled-domain » fut supporté par plusieurs expériences *in vivo* et *in vitro*. Wu *et al.* (1988) démontrèrent l'accumulation de supertours positifs dans le plasmide pBR322, lorsqu'il est extrait d'une souche *E. coli* traitée à la novobiocine (un inhibiteur de la gyrase). La formation de ces supertours dépendaient directement de la transcription, puisque l'ajout de rifampicine empêchait leur accumulation. De plus, un plasmide témoin montra clairement que l'expression du gène *tetA* contribuait significativement à la production de supertours positifs. Ce plasmide témoin, le pBR322 ΔP*tetA*, est identique au pBR322 à l'exception du promoteur du gène *tetA* qui porte une petite délétion dans la région -10, ce qui inactive l'expression de *tetA*. Les auteurs remarquèrent aussi que la transcription du gène *bla*, orienté de manière divergente avec *tetA*, amplifiait significativement l'accumulation des supertours positifs. Par la suite, d'autres données *in vitro* provenant du même laboratoire supportèrent le modèle du « twin-supercoiled-domain » (Tsao *et al.*, 1989). Les résultats de ces travaux

suggérèrent que la quantité de supertours qui s'accumule est directement proportionnelle à la longueur du transcrit, de même que la vitesse de la polymérase ARN. Selon ces données, les domaines de surenroulement créés devant et derrière la polymérase, dépendent directement de la force que la polymérase exerce sur l'ADN. Plus le transcrit sera long et plus la vitesse de la polymérase sera élevée, plus la tension dans l'ADN sera considérable.

Selon ce modèle, dans certains contextes particuliers de transcription, comme l'attachement à la membrane de la protéine exprimée, la production de supertours atteindra un degré plus élevé. Plusieurs études ont démontré que la partie amino-terminale de la protéine TetA était essentielle à la production de plasmides hypernégativement surenroulés extraits de mutants *topA* (Lodge *et al.*, 1989; Lynch and Wang, 1993). Cette séquence d'environ 98 aa, contient trois régions hydrophobes dont le rôle probable est l'attachement à la membrane cellulaire. Lynch et Wang (1993) ont d'ailleurs montré que la production de topoisomères hypernégatifs chez les mutants *topA* était un phénomène général (non unique à *tetA*) relié à l'expression de protéines membranaires (*lacY*, *melB*) ou destinées à l'exportation (*tolC*, *ampC*). Il est intéressant de mentionner que le produit du gène *bla* de pBR322 est exporté dans l'espace périplasmique de manière post-traductionelle (Koshland and Botstein, 1982), ce qui explique l'absence d'effet de sa transcription sur le surenroulement des plasmides.

D'autres contextes de transcription différents de celui du gène *tetA* vont aussi mener à la génération des topoisomères hautement surenroulés. Par exemple, l'inactivation de la topoisomérase I chez *Saccharomyces cerevisiae* provoque la formation de plasmides hypernégativement surenroulés (Brill and Sternglanz, 1988). Comme la transcription et la traduction ne sont pas couplées chez les eucaryotes, l'ancrage à la membrane durant la transcription ne peut expliquer ce résultat. Chez les bactéries, l'activation en *cis* de la transcription du gène *galK* porté par un plasmide rendra celui-ci hypernégatif, malgré l'absence d'ancrage à la membrane (Franco and Drlica, 1989). Donc, l'ancrage à la membrane durant l'élongation de la transcription

n'est pas une condition absolue de formation de plasmides hypernégativement surenroulés dans un mutant *topA*.

6.2.4 Régulation du surenroulement de l'ADN bactérien

Les mutants *topA* de *E. coli* démontrent une importante diminution de leur taux de croissance et, en général, pour qu'ils puissent croître, ils doivent accumuler des mutations compensatoires affectant, le plus souvent, l'activité de la gyrase (DiNardo *et al.*, 1982; Pruss *et al.*, 1982; Raji *et al.*, 1985). Ces mutations compensatoires surviennent spontanément et permettent à la cellule mutante de croître presque aussi rapidement que la souche sauvage. Un exemple de mutation compensatoire est l'allèle *gyrB225* de la souche DM800 [Δ(*topA cysB*) *gyrB225*]. Cette souche demeure viable malgré l'insertion d'un allèle *gyrB* sauvage, ce qui suggère qu'elle possède, ailleurs dans son génome, au moins une seconde mutation compensatoire (DiNardo *et al.*, 1982).

On considère que la gyrase et la topoisomérase I, grâce à leur activité opposées, créent un état d'équilibre topologique dans le surenroulement de l'ADN chromosomique bactérien. Ce modèle s'appuie aussi sur le fait que l'expression des gènes de la topoisomérase I et de la gyrase est régulée de manière homéostatique. Le surenroulement négatif dans l'ADN active le gène de la topoisomérase I (Tse-Dinh *et al.*, 1988) tandis que l'ADN relaxé induit la synthèse de la gyrase (Menzel *et al.*, 1983). Lorsque les cellules sont traitées avec la chloroquine, qui s'intercale entre les nucléotides et relaxe l'ADN, on observe alors l'introduction de nouveaux supertours négatifs par la gyrase (Esposito and Sinden, 1987). Le modèle classique de la régulation du surenroulement de l'ADN propose que lorsque l'activité de relaxation de l'ADN par la topoisomérase I est absente, le chromosome devient trop surenroulé négativement, par l'action de la gyrase, pour permettre la viabilité de la cellule. Ainsi, selon les résultats d'études *in vivo*, la croissance bactérienne ne s'effectuerait que dans une variation du surenroulement global de l'ADN de l'ordre de $\pm15\%$ (Drlica, 1992).

6.2.5 Régulation du surenroulement global par la gyrase

La gyrase utilise l'hydolyse d'ATP pour pouvoir introduire des supertours négatifs. Néanmoins, des études récentes ont démontré que la gyrase pouvait interagir avec l'analogue non hydrolysable de l'ATP, l'ADPNP (5'-adenylyl-β-γ-imidophosphate) et introduire un tour de surenroulement (Tamura and Gellert, 1990; Tamura *et al.*, 1992). L'hydrolyse de l'ATP ne servirait qu'à libérer les produits du site actif de l'enzyme (Sugino *et al.*, 1978) pour ensuite recommencer le cycle. La gyrase peut aussi relaxer l'ADN surenroulé positivement selon une réaction dépendante de l'hydrolyse d'ATP équivalente à l'introduction de supertours négatifs. Il est intéressant de noter qu'en absence d'ATP, la gyrase peut relaxer l'ADN surenroulé négativement (Bates and Maxwell, 1989; Wigley *et al.*, 1991). Hsieh *et al.* (1991) ont déterminé que le ratio intracellulaire [ATP]/[ADP] et le surenroulement de l'ADN semblaient intimement reliés. En faisant varier le ratio [ATP]/[ADP], il a été possible de moduler les activités de surenroulement et de relaxation de la gyrase. En effet, dans des conditions où le ratio [ATP]/[ADP] augmente, le surenroulement négatif de l'ADN augmente également. Grâce à ces données, les auteurs ont suggéré un modèle de régulation de la densité de surenroulement globale de l'ADN par la gyrase, via le ratio [ATP]/[ADP].

6.2.6 La formation de R-loops durant la transcription chez *E. coli*

Dans un contexte d'élongation de la transcription tel que décrit précédemment, il est normal de déduire que le rôle principal de la topoisomérase I est d'atténuer la formation locale de supertours négatifs. Ces résultats supportent le modèle du « twin-supercoiled-domain » et s'opposent au modèle classique où l'enzyme doit uniquement veiller à garder le surenroulement global de l'ADN à un niveau déterminé. Étant donné que les mutants *topA* utilisés dans les expériences ayant servi à démontrer le « twin-supercoiled-domain » ont une croissance pratiquement normale (souche DM800), on peut en conclure que l'élimination des supertours négatifs, générés durant la transcription, n'est pas essentielle à la viabilité de la cellule. Il est donc vraisemblable que les résultats des expériences décrites plus haut (production de

topoisomères hypernégativement surenroulés) ne reflètent pas la fonction essentielle de la topoisomérase I.

La transcription *in vitro* du plasmide pBR322 par la polymérase ARN de *E. coli* en présence de gyrase, génère des topoisomères hypernégativement surenroulés (Drolet *et al.*, 1994a). La formation de ce type de topoisomères montre une sensibilité à la RNase H, suggérant la formation de R-loops durant la transcription. Ces expériences de transcription soulèvent l'hypothèse que le surenroulement local généré durant l'élongation de la transcription, est impliqué dans la formation des R-loops. L'activité de la gyrase favorise la production de plasmides hypernégativement surenroulés durant l'élongation de la transcription. Ces plasmides hautement surenroulés représentent une population de topoisomères avec un niveau de surenroulement négatif excessivement élevé que la gyrase, à elle seule, ne peut introduire. L'accumulation de ce type particulier de topoisomères dépend entièrement de la présence d'un hybride ARN-ADN formé durant l'élongation de la transcription. Plus l'hybride s'allonge, plus le plasmide devient relaxé, alors la gyrase introduit de nouveaux supertours négatifs, favorisant encore l'allongement de l'hybride. Lorsque l'ARN est éliminé de la matrice (lors de l'extraction), le surenroulement contraint par l'hybride se trouve libéré et le plasmide devient alors hypernégativement surenroulé. Dans ce travail, les auteurs ont remarqué que la présence de la RNase A dans la réaction pouvait aussi éliminer la formation de topoisomères hautement surenroulés. Ceci laissait supposer un mécanisme de formation de R-loops dans lequel l'ARN nouvellement transcrit devait se trouver déplacé, à un moment ou à un autre.

Ces observations ont été supportées par des données *in vivo* obtenues en utilisant des mutants *topA gyrB*(Ts). Ces souches se comportent comme de vrais mutants *topA* lorsqu'elles sont incubées à température restrictive, et elles démontrent alors une croissance fortement diminuée qui peut être partiellement corrigée par la surproduction de la RNase H. Ces résultats proviennent du travail réalisé à l'aide de deux mutants *topA* de *E. coli*; un ayant le génotype Δ(*topA cysB*) *gyrB203*(Ts) (souche RFM475), et l'autre, portant le génotype *topA*::Tn*10 gyrB203*(Ts) (souche RFM480). Les deux souches sont isogéniques mis à part l'allèle *topA*. Le premier

mutant contient une délétion du gène *topA* et du gène voisin *cysB*, alors que le deuxième mutant possède une insertion du transposon Tn*10* dans le gène *topA*, qui rend le produit inactif. Grâce à l'allèle *gyrB* sensible à la température, il est possible de travailler avec de vrais mutants *topA* dans certaines conditions spécifiques de croissance (Drolet *et al.*, 1995). Lorsque les cellules sont incubées à la température de 37°C, la gyrase est suffisamment inactive pour compenser la perte de *topA* et permettre la croissance, alors qu'à 28°C, son activité est trop forte et la croissance est inhibée. C'est aussi à cette température restrictive que la surproduction de la RNase H démontre une forte stimulation de la croissance. L'explication la plus simple est que lorsque la gyrase retrouve son activité enzymatique sauvage, elle favorise la formation et l'élongation d'hybrides ARN-ADN en absence de la topoisomérase I. Ces structures empêchent la croissance des cellules à moins qu'elles ne soient éliminées par la RNase H.

Selon ces données, l'absence de la topoisomérase I aurait pour conséquence la formation de R-loops, qui seraient néfastes pour la cellule. Le lien fonctionnel entre la topoisomérase I et la RNase H est d'autant plus fort que le double mutant *topA rnhA* (gène codant pour la RNase H) n'est pas viable. De plus, la quantité de RNase H nécessaire à la croissance augmente en fonction de l'activité intracellulaire de la gyrase (Drolet *et al.*, 1995). Ces nouveaux résultats permettent de suggérer que, dans un mutant *topA*, les mutations compensatoires acquises dans la gyrase ne surviennent pas dans le but de diminuer le niveau de surenroulement de l'ADN, mais plutôt pour réduire la formation de R-loops. Cette hypothèse est d'autant plus plausible que l'introduction de mutations diminuant l'activité de la gyrase favorise la croissance de mutants *rnhA* (Drolet *et al.*, 1995).

6.2.7 Modèle de régulation de la formation des R-loops

À partir de ces résultats *in vivo*, de concert avec les résultats *in vitro* présentés plus haut, un modèle de régulation de la formation des hybrides ARN-ADN a été élaboré. La formation de R-loops lors de la transcription serait réprimée par la RNase H ou la topoisomérase I, cette dernière, en relaxant l'ADN, déstabiliserait la structure

hybride, tandis que la gyrase favoriserait leur formation en introduisant des supertours négatifs (Voir Figure 2; Drolet *et al.*, 1994a; 1995).
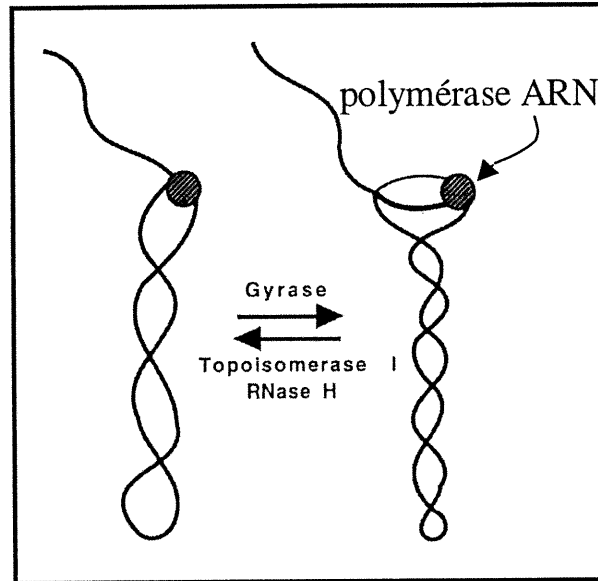
Figure 2. Modèle de la formation de R-loops durant l'élongation de la transcription chez *E. coli*. La gyrase favorise l'élongation de l'hybride alors que la RNase H et la topoisomérase I les élimine. Tiré de Drolet *et al.*, (1994a).

6.2.8   Le substrat de la topoisomérase I

On sait depuis longtemps que, *in vitro* et *in vivo*, la topoisomérase I ne relaxe pas de manière efficace et complète l'ADN surenroulé négativement (Wang, 1971; Pruss *et al.*, 1986; Bliska and Cozzarelli, 1987). De plus, il apparaît que la spécificité de l'enzyme pour l'ADN surenroulé négativement, découle du fait qu'elle requiert une région ADN simple-brin flanquée d'une région double-brin afin de former un complexe enzyme-substrat actif (Kirkegaard and Wang, 1985). Cette observation est renforcée par les essais de relaxation *in vitro* par la topoisomérase I, qui se montre beaucoup plus efficace lorsque le plasmide contient un R-loop (Phoenix *et al.*, 1997). D'ailleurs, ce résultat supporte bien le modèle proposé que le rôle principal de la topoisomérase I est d'empêcher la formation de R-loops. En effet, la relaxation de l'ADN déstabilise les hybrides ARN-ADN.

6.2.9   La tension de surenroulement et le surenroulement contraint

Fait important à noter, le surenroulement intracellulaire de l'ADN se retrouve sous deux formes, la tension de surenroulement et le surenroulement contraint. Ces deux types de surenroulements sont mis en évidence à l'aide d'une expérience assez simple. L'introduction d'une cassure simple-brin, *in vivo* et *in vitro*, dans une molécule circulaire, permet la rotation des brins et la relaxation de cette molécule (la tension de surenroulement) (Sinden *et al.*, 1980). Cependant l'ouverture et la ligation *in vivo* de cette cassure simple-brin ne libère seulement que la moitié du surenroulement, alors que cette libération est complète *in vitro* (Pettijohn and Pfenninger, 1980). La portion non libérée de surenroulement (contraint) serait vraisemblablement contrainte par les protéines de type histone (ex. HU) ainsi que les polymérases ARN (Drlica, 1992).

Albert *et al.* (1996) ont démontré que le surenroulement hypernégatif formé dans un mutant *topA* par la transcription du gène *tetA* situé sur un plasmide, est majoritairement contraint à l'intérieur de la cellule. Les auteurs ont utilisé un segment d'ADN pouvant former une transition graduelle de structure B à Z, en fonction de l'augmentation de la densité de surenroulement négative. On peut mesurer

directement le taux de transition de ce segment à l'aide du OsO_4. Le segment de transition, située en amont d'un promoteur *tetA*, se trouve fusionné soit à un gène *tetA* ou *cat*. Ces constructions permettent de quantifier la tension de surenroulement durant la transcription de *tetA*, reliée à l'ancrage de la protéine Tet à la membrane, et de le comparer avec la tension généré par le gène *cat*, dont le produit est cytoplasmique. Les résultats ont indiqué que dans un mutant *topA*, la tension de surenroulement produite était semblable pour les deux gènes. Cependant, seulement l'expression du gène *tetA* mène à la production de topoisomères hypernégatifs. Il est donc très probable que l'excès de surenroulement négatif lié à l'attachement à la membrane soit contraint dans la cellule, puisque celui-ci n'est pas détecté *in vivo*. Les auteurs expliquent ces résultats en évoquant la présence de R-loops sur ces plasmides, tel que proposé auparavant (Drolet *et al.*, 1994a; 1995).

6.3 *Les opérons ribosomaux*

6.3.1 Sites potentiels de formation de R-loops

La formation de R-loops semble être une conséquence importante de l'absence de la topoisomérase I durant l'élongation de la transcription puisque cette structure est apparemment responsable de l'inhibition de croissance des mutants *topA*. Cependant, les études précédentes ne se sont pas penchées sur la fréquence ou les sites de formation de ces structures. Plusieurs caractéristiques spécifiques aux opérons ribosomaux favorisent l'hypothèse qu'ils puissent être des sites privilégiés de formation de R-loops.

Chez les procaryotes, la traduction des ARNm est couplée à la transcription. Cette particularité pourrait permettre d'empêcher l'appariement de l'ARNm naissant avec la matrice ADN. Par ailleurs, le transcrit des opérons ribosomaux n'étant pas traduit, il peut plus aisément s'apparier avec la matrice ADN. Dans cet ordre d'idée, il est intéressant de mentionner que les mutants *topA* (Drolet *et al.*, 1995) et *rnhA* (Kogoma *et al.*, 1993) sont sensibles aux « shifts-up » nutritionnels. Il a été démontré que c'est dans ces conditions de croissance que la synthèse en ARNr est la plus forte (Condon *et al.*, 1995a), et donc que la formation de R-loops inhibiteurs est la plus

propice. Finalement, les doubles mutants *nusB5 topA* et *nusB5 rnhA* sont très peu viables (E. Massé et M. Drolet, résultats non publiés). Le gène *nusB* code pour un facteur d'antiterminaison de la transcription important pour l'expression des opérons *rrn* (voir section 6.3.5).

6.3.2 Organisation des opérons *rrn*

La bactérie *E. coli* possède sept opérons ribosomaux distribués de manière asymétriques sur une moitié du chromosome. Ils sont tous orientés pour que leur transcription et la fourche de réplication puissent évoluer dans la même direction. Chaque opéron code pour un seul ARN précurseur, qui, une fois clivé par des RNases spécifiques, produira trois ARN ribosomaux (ARNr) ainsi que un à trois ARN de transfert (ARNt). Les ARNr sont disposés sur le transcrit respectivement dans l'ordre (à partir du promoteur) 16S, 23S et 5S. La présence de ces trois gènes transcrits sur le même opéron permet une production équimolaire des trois espèces d'ARNr. Ceux-ci iront former les deux sous-unités 30S (16S + 21 polypeptides) et 50S (23S + 5S + 34 polypeptides) d'un ribosome. La transcription des opérons ribosomaux peut être très exceptionnelle; en condition de croissance rapide (phase exponentielle en milieu riche), plus de 50% de l'activité transcriptionnelle de la cellule sert à synthétiser les ARNr (Condon *et al.*, 1995b).

6.3.3 Régulation de l'expression des opérons ribosomaux

La synthèse de ribosomes augmente approximativement selon le carré du taux de croissance. L'ARN ribosomal est le facteur limitant dans la synthèse de ribosomes, puisque les protéines ribosomales sont régulées par la concentration en ARNr (Nomura *et al.*, 1984; Zengel and Lindahl, 1994). La transcription de chacun des sept opérons *rrn* de *E. coli* est initié à partir de deux promoteurs, P1 et P2. La plupart des transcrits proviennent du promoteur P1, dont l'activité augmente avec le carré du taux de croissance (Gourse *et al.*, 1986). Plusieurs régulateurs affectent l'activité de P1. Les régulateurs positifs sont les suivants : l'élément UP, en amont des promoteurs, qui augmente l'activité de P1 en interagissant avec la sous-unité α de la polymérase ARN (Blatter *et al.*, 1994); le facteur de transcription FIS qui s'attache à plusieurs

sites en amont de l'élément UP et qui interagit directement avec la polymérase ARN (Bokal *et al.*, 1997); les facteurs d'antiterminaisons qui, en interagissant avec la séquence BoxA, empêchent la transcription de terminer prématurément (voir plus loin). Un régulateur négatif de l'expression des opérons *rrn*, le ppGpp, est synthétisé lors d'une diminution de la concentration en acides aminés (« stringent response »; Josaitis *et al.*, 1995).

Le taux de croissance cellulaire varie en relation avec la disponibilité des nutriments. Afin qu'elle ne produise pas plus de ribosomes qu'il n'est nécessaire, la cellule a la capacité de réguler la formation de ribosomes. Par exemple, l'expression des protéines ribosomales répond à la disponibilité en ARNr afin de produire des ribosomes (Keener and Nomura, 1996). Lorsqu'une protéine s'accumule en excès, elle interagit avec son ARNm correspondant et inhibe sa traduction. Le modèle de régulation de la synthèse des ARNr, proposé par Jinks-Roberston *et al.* (1983), suggère que le modulateur central soit les ribosomes libres, donc non actif. L'ajout d'opérons ribosomaux diminue l'expression de chacun de ces derniers et, inversement, la délétion d'opérons ribosomaux augmente l'expression des opérons restants (Condon *et al.*, 1993). Cependant, l'élément modulateur ne serait pas le ribosome libre mais serait plutôt la capacité de synthèse protéique de la cellule (Jinks-Robertson *et al.*, 1983; Cole *et al.*, 1987). Ainsi, la cellule garderait un réservoir précis de ribosomes en jaugeant la capacité de synthèse protéique de ce pool et non sa concentration en ribosomes. Les résultats récents de Gaal *et al.* (1997) ont, en effet, démontré qu'une concentration élevée de nucléotides initiateurs de la transcription des opérons *rrn* (ATP ou GTP) permettait d'initier plus fréquemment et aussi de stabiliser le complexe de transcription au niveau des promoteurs des opérons *rrn*, donc de produire plus d'ARNr. Les auteurs ont proposé un modèle où la concentration intracellulaire d'ATP et de GTP (déterminée par le taux de synthèse et de consommation de ces deux nucléotides) contrôlerait la production d'ARNr. La synthèse d'ATP et de GTP est déterminée par les conditions de croissance alors que leur consommation dépend du taux de synthèse protéique.

Les opérons ribosomaux ont deux promoteurs principaux, P1 et P2, dont les transcrits traversent un site, localisé environ à 10 pb en aval du promoteur P2, qui favorise l'antiterminaison *in vivo* (Li *et al.*, 1984). Une autre séquence d'antiterminaison se retrouve dans le segment espaceur entre les gènes 16S et 23S suggérant que le système doit être rechargé après avoir traversé le fragment 16S (Berg *et al.*, 1989). Ce système fut suggéré après la découverte d'un terminateur dépendant du facteur Rho, situé dans le fragment *Hind*III du segment 16S, qui pourtant, n'avait aucun effet sur la production d'ARNr (Askoy *et al.*, 1983). Ces sites d'antiterminaison des opérons *rrn* contiennent des domaines appelés *boxA*, *boxB* et *boxC*, qui partagent beaucoup de similitudes avec la séquence d'antiterminaison *nut* (« N utilization ») du phage lambda (Olson *et al.*, 1982). Chez le phage lambda, le complexe d'antiterminaison modifie la polymérase ARN de l'hôte (*E. coli*) à l'aide de la protéine phagique N, de concert avec les protéines bactériennes telles NusA, NusB, NusE et NusG. L'assemblage de ces protéines requiert le site *nut* de l'ARN, situé à proximité du promoteur. Apparemment, le segment *nut* de l'ARN serait le noyau du complexe d'antiterminaison une fois ce dernier assemblé. Ainsi, la partie 5' de l'ARN nouvellement transcrit resterait attaché à la polymérase ARN durant la transcription du gène (Mason *et al.*, 1992). Cette combinaison produit un complexe d'élongation capable de résister à de nombreux terminateurs de transcription localisés sur plusieurs kb de distance (Richardson and Greenblatt, 1996).

6.3.4 La terminaison de la transcription

La terminaison de la transcription se produit lorsque l'ARN naissant est relâché du complexe qu'il forme avec la polymérase ARN et l'ADN. Ceci peut se produire spontanément ou à l'aide d'un facteur intermédiaire. Le premier type de terminateur se nomme intrinsèque et le second, impliquant le facteur Rho, se nomme Rho-dépendant (Richardson and Richardson, 1996). Le site de terminaison intrinsèque possède un segment G+C riche capable de former une structure tige-boucle très stable dans l'ARN nouvellement transcrit (Cheng *et al.*, 1991). Cette structure contribuerait à affaiblir l'interaction entre l'ARN naissant et le complexe de transcription (Richardson and Greenblatt, 1996). Une deuxième particularité des terminateurs

intrinsèques est la présence de segments riches en adénosine dans le brin ADN matrice, peu après la structure tige-boucle (Lynn *et al.*, 1988). L'interaction A-U est plutôt faible comparé à la plupart des autres interactions possibles dans une hélice hybride, ceci pourrait donc aider à la déstabilisation de la polymérase ARN.

Le second type de site de terminateur, Rho-dépendant, possède deux segments importants situés à environ 150-200 pb l'un de l'autre. Un des segment est le site où la terminaison se produit, le site d'arrêt de la transcription, *tsp* (« transcription stop point »). Le second segment est le site d'attachement de la protéine Rho à l'ARN, *rut* (« Rho-utilization »). Ces deux segments se distribuent sur 150-200 pb, avec le segment *rut* en amont du site d'arrêt de la transcription (Richardson and Greenblatt, 1996). La protéine Rho commence par s'attacher à l'ARN nouvellement transcrit au site *rut*, riche en cytosine. Par la suite, elle utilise son activité ATPase pour se déplacer vers l'extrémité 3' de l'ARN, jusqu'à l'endroit où la polymérase est arrêtée au site *tsp* (Richardson and Richardson, 1996). Rho catalyse alors la dissociation de la polymérase ARN grâce à l'activité hélicase ARN-ADN qui lui permet probablement de détacher l'ARN situé dans la bulle de transcription (Brennan *et al.*, 1987). Apparemment, Rho garde un contact continu avec le segment *rut* durant la dissociation de l'ARN en 3', ce qui provoque la formation d'une boucle dans l'ARN (Steinmetz and Platt, 1994).

6.3.5   L'antiterminaison

Les premières évidences expérimentales reliées à l'existence d'un système d'antiterminaison chez les opérons ribosomaux proviennent de la démonstration de séquence *nut* similaire au phage lambda, situées dans les régions de régulation (Li *et al.*, 1984). Peu de temps après, Sharrock *et al.* (1985) montraient que certains facteurs Nus de la bactérie semblaient impliqués dans l'antiterminaison des opérons *rrn*. Dans ce travail, les auteurs ont remarqué que chez le mutant *nusB5* de *E. coli*, la transcription des opérons *rrn* pouvait se terminer prématurément. Chez une souche de génotype sauvage, près de la totalité des transcrits initiés sur un opéron *rrn* termineront adéquatement, produisant ainsi un ratio équivalent (1:1:1) de ses différents ARN ribosomaux (16S, 23S et 5S). Chez le mutant *nusB5* cependant,

environ 40% des transcrits terminent prématurément, ce qui diminue la production des ARNr distaux. Afin de compenser pour cette perte, le taux d'initiation de la transcription des opérons *rrn* du mutant *nusB5* augmente de 1,5 fois, produisant alors un nombre normal de ribosomes. Les travaux de Gourse *et al.* (1986) ont démontré que la séquence *boxA* des opérons ribosomaux était nécessaire et suffisante afin que la polymérase ARN puisse traverser un terminateur Rho-dépendant fort. Le rôle de NusB dans l'antiterminaison fut démontré plus directement par Squires *et al.* (1993). Ceux-ci démontrèrent que l'ajout de la protéine NusB dans un extrait cellulaire dans lequel ce facteur est absent, était nécessaire à l'antiterminaison *in vitro*. Des études génétiques et biochimiques ont montré que NusB et NusE interagissaient ensemble pour former un hétérodimère (Mason *et al.*, 1992). La protéine NusE se lierait à la polymérase ARN, ce qui aurait comme conséquence la formation d'une boucle dans le transcrit (Mason and Greenblatt,1991; Greenblatt *et al.*, 1993). Enfin, d'autres facteurs essentiels, encore inconnus, seraient également impliqués dans l'élaboration complète du complexe de transcription résistant aux terminateurs des opérons *rrn* (Squires *et al.*, 1993). Chez le phage lambda, les interactions *boxA*-NusB-NusE-polymérase ARN permettent de transcrire au travers des terminateurs sur plusieurs kb après le site *nut.* (DeVito and Das, 1994).

Un modèle expliquant le mécanisme de l'antiterminaison est basé sur l'observation que le taux de transcription de la polymérase ARN est accéléré d'environ 2 fois (80 nt/sec pour l'ARNr contre 40 nt/sec pour l'ARNm; Vogel and Jensen, 1997). Ainsi, la vitesse de la polymérase deviendrait trop élevée pour que la protéine Rho puisse rejoindre et déstabiliser le complexe de transcription (Jin *et al.*, 1992).

Le système d'antiterminaison des opérons *rrn* de *E. coli* ne fonctionne qu'avec les terminateurs Rho-dépendants (Albrechtsen *et al.*, 1990), alors que le système d'antiterminaison de lambda est également résistant aux terminateurs intrinsèques (Das, 1993).

6.3.6 Formation de R-loops durant la transcription des opérons *rrn*

Si la transcription des opérons ribosomaux provoque la formation de R-loops, une augmentation du taux de transcription de ceux-ci pourrait amplifier les problèmes reliés à la présence d'hybrides. L'introduction du plasmide pNO1302, portant un opéron *rrnB* incomplet, permet effectivement d'augmenter la transcription sans modifier le réservoir de ribosomes intracellulaire. Dans cette construction dérivée de pBR322, l'opéron porte une délétion partielle des gènes codant pour les sous-unités 16S et 23S, qui ne peuvent produire d'ARNr fonctionnels. L'introduction dans un mutant *topA* du plasmide pNO1302 provoque une inhibition complète de la croissance (Massé *et al.*, 1997). On ne peut transformer ce plasmide, dans une souche *topA*, que si la RNase H est surproduite ou si les deux promoteurs de l'opéron *rrnB* sont absents.

Les données précédentes nous permettaient de supposer qu'un fragment transcrit de l'opéron *rrnB* favorisait la production de R-loops dans un mutant *topA*. Afin d'étudier le rôle des topoisomérases dans la régulation de la formation de R-loops, le fragment *Hind*III (567 pb; présent sur le pNO1302) du segment 16S de l'opéron *rrnB* fut cloné dans un vecteur pBluescript. Ce vecteur permet la transcription du même fragment ADN dans les deux directions, grâce aux promoteurs spécifiques aux polymérases ARN des phages T3 et T7 orientés de manière convergente. La transcription *in vitro* de ce fragment par les polymérases des phages T3 et T7 en présence de gyrase, montre la formation de topoisomères hypernégativement surenroulés. Ces topoisomères sont directement dépendants de la formation de R-loops puisque l'ajout de la RNase H dans la réaction élimine totalement leur production. Il fut aussi noté que la formation de topoisomères hypernégatifs était beaucoup plus résistante à la RNase A lorsque le fragment était transcrit dans l'orientation physiologique que le témoin inverse (Phoenix *et al.*, 1997). Ceci démontre que le R-loop, pour des raisons encore inconnues, est plus stable lorsque le fragment est transcrit dans son orientation physiologique. De plus, cette résistance à la RNase A suggère que l'ARN n'est jamais déplacé de la matrice tout au long de son élongation. Dans ce système utilisant les polymérases ARN de phages,

l'hybride serait donc formé du coté 5' vers 3'. Des travaux récents sur la polymérase du phage T7 ont suggérés que le déplacement de l'ARN naissant du brin matrice ADN était dépendant, en partie, de la force de réappariement de la matrice, de même que de l'interaction dans l'hybride ARN-ADN (Gopal *et al.*, 1999). La transcription sur une matrice linéaire permet le déplacement complet de l'ARN naissant, alors que sur une matrice surenroulée négativement, l'ARN tend à rester hybridé au brin matrice. Ces données sont supportées par d'autres résultats de transcription *in vitro*, provenant de notre laboratoire, à l'aide des polymérases ARN des phages T3 et T7 (Phoenix *et al.*, 1997).

6.4   Fonctions biologiques des R-loops

6.4.1   L'origine de réplication plasmidique ColE1

La réplication du plasmide pBR322 à partir de son origine ColE1 ne requiert aucune protéine codée par le plasmide lui-même. Seulement trois enzymes sont nécessaires afin d'initier la synthèse du brin « leader », soit la polymérase ARN, la polymérase ADN I et la RNase H (Itoh and Tomizawa, 1982). La polymérase ARN transcrit un fragment d'ARN (ARN II) débutant à 555 pb en amont du point considéré comme étant l'origine de réplication (Itoh and Tomizawa, 1980). Ce transcrit forme une structure complexe qui résulte en un hybride ARN-ADN stable dans sa propre région 3'. Masakuta et Tomizawa (1990) ont démontré que cette structure formée par le brin ARN de 555 pb était critique à la formation d'une origine fonctionnelle. Il a été déterminé *in vitro* qu'une concentration sous optimale de ribonucléotides dans la réaction de transcription aboutissait plus fréquemment à la formation de R-loop initiateur de la réplication (Masakuta and Tomizawa, 1990). Ceci suggère que la vitesse de transcription influence grandement l'appariement de l'ARN II au site d'initiation de la réplication. Une polymérase ARN moins rapide permet peut-être la formation de la structure secondaire dans l'ARN II nécessaire pour l'appariement de l'ARN à l'origine. Dans le même ordre d'idée, un site de pause très fort a été localisé à 7 pb en amont de l'origine (Tomizawa and Itoh, 1982). Ce site de pause pourrait permettre à l'ARN II de compléter son repliement et, ainsi, d'augmenter l'efficacité de la formation de l'hybride.

Une fois l'hybride formé, l'enzyme RNase H coupe à l'intérieur du brin ARN afin de générer une extrémité 3' OH compétente à l'initiation de la réplication du brin continu par la polymérase ADN I. Après la synthèse de quelques 400 nucléotides, la polymérase ADN III, beaucoup plus efficace, prend le relais. Ce transfert de polymérase ADN pourrait permettre d'initier la réplication du brin ADN opposé, ce qui apporterait l'activité hélicase nécessaire à la réplication discontinue (Baker and Kornberg, 1992). Ce site d'échange de polymérases et d'initiation de la synthèse du brin complémentaire s'appelle *pas* pour "*primosome assembly site*". Le primosome se compose des protéines PriA, PriB, PriC, DnaB, DnaC, DnaT, SSB ainsi que la primase (DnaG), en plus des protéines déjà présentes dans le complexe de synthèse du brin continu. La réplication est unidirectionnelle et les deux brins (continu et semi-continu) sont synthétisés quasi simultanément.

Cependant, les plasmides portant l'origine de réplication ColE1 peuvent se répliquer adéquatement dans un mutant RNase H, ce qui est en contradiction avec les expériences *in vitro* (Naito *et al.*, 1984). Dans ces conditions, la polymérase ADN I n'est plus essentielle (Kogoma, 1984). Ceci démontre bien qu'il existe un mécanisme alternatif aboutissant à l'initiation de la réplication, qui néanmoins, nécessite toujours la formation de l'hybride à l'origine de réplication (Dasgupta *et al.*, 1987). En effet, en absence de RNase H, il a été montré que l'attachement de l'ARN II à l'ADN de la région ColE1 déplace le brin ADN non transcrit (complémentaire), ce qui le rend disponible pour l'initiation de la réplication discontinue (Masakuta *et al.*, 1987). Ainsi, le brin ARN II hybridé n'est pas utilisé comme amorce et la longueur de l'hybride semble critique dans le succès de l'initiation de la réplication. Les auteurs ont déterminé qu'un hybride d'une longueur d'au moins 40 nucléotides était nécessaire pour activer la réplication.

6.4.2 Origine de réplication *oriC* de *E. coli*

La réplication du chromosome de *E. coli* débute à partir d'une seule et unique origine de réplication appelée *oriC*. Elle a été localisée à la 84ième min. sur le chromosome. L'activation de la réplication à *oriC* semble intimement liée à l'action de la polymérase ARN, puisque la présence de rifampicine inhibe l'initiation de la

réplication (Lark, 1972). De plus, certaines mutations dans les sous-unités β et β' de la polymérase ARN affectent le nombre de copie des plasmides *oriC* (minichromosomes) ainsi que du chromosome (Rasmussen *et al.*, 1983). D'autres mutations dans la sous-unité β de la polymérase ARN corrigent le phénotype de sensibilité à la température d'un allèle mutant de *dnaA*, dont le produit est essentiel pour initier la réplication (Atlung, 1984; Bagdasarian *et al.*, 1977). Plusieurs promoteurs se trouvent dans la région minimale de *oriC*, mesurant 245 pb, mais leur rôle dans l'activation de la réplication demeure obscur. Dans cette région, le gène *mioC* (« modulation of initation at *oriC* ») produit une protéine de 16 kDa dont le rôle demeure inconnu. La transcription de ce gène se fait en direction de *oriC* et se termine à plusieurs endroits à l'intérieur même de l'origine et un peu plus loin (Junker *et al.*, 1986; Rockeach and Zyskind, 1986; Schauzu *et al.*, 1987). Il est possible que l'ARN produit puisse servir d'amorce pour la synthèse d'ADN puisque plusieurs des sites de terminaisons de transcription coïncident avec des jonctions ARN-ADN (Hirose *et al.*, 1983; Kohara *et al.*, 1985). Comme il a été observé que la délétion du gène *mioC* n'empêchait pas la réplication dépendante à *oriC*, il ne peut être relié directement au phénotype de sensibilité à la rifampicine lors de l'initiation de la réplication. Dans un système reconstitué de protéines purifiées, l'initiation de la réplication à *oriC* ne nécessite pas toujours la présence de la polymérase ARN (Ogawa *et al.*, 1985; van der Ende *et al.*, 1985). Cependant, lorsque la quantité de protéine HU ou de topoisomérase I est trop élevée ou lorsque la température n'est pas favorable, l'ajout de polymérase ARN devient alors essentiel à l'initiation. Cette dernière agirait bien avant la formation de l'amorce, ce qui suggère qu'elle ne serait pas directement impliquée dans l'initiation mais plutôt dans l'activation de l'origine. En effet, dans des conditions de densité de surenroulement défavorable, la formation d'un R-loop dans la région de *oriC* aiderait à déclencher l'initiation de la réplication (Baker and Kornberg, 1988). Si le surenroulement de l'ADN se trouve partiellement relaxé (densité de surenroulement moins négative que -0.03), la réplication ne pourra être initiée, à moins qu'un R-loop ne soit déjà présent sur la matrice, ou éventuellement généré par la transcription (Baker and Kornberg, 1988). Ce surenroulement défavorable est comparable à celui que l'on retrouve dans la cellule (-0.025;

Pettijohn, 1988). L'ARN présent dans la structure R-loop ne joue pas le rôle d'amorce puisque la présence d'extrémité 3' OH, nécessaire pour amorcer la polymérase ADN, est facultative. Son site de formation n'est pas critique et, il peut activer *oriC* sur une distance pouvant aller jusqu'à 1.4 kb. Il est intéressant de mentionner que la présence de séquences ADN riches en G et C situées entre le R-loop et l'origine *oriC* peut inhiber l'activation (Skarstad *et al*, 1990). Ceci démontre que le R-loop agit probablement en aidant la séparation des brins d'ADN dans la région *oriC*. La présence d'un segment riche en G et C entre l'origine et le R-loop empêche ce dernier de propager son énergie car ces séquences exigent plus d'énergie afin qu'elles puissent se séparer (Breslauer *et al.*, 1986).

6.4.3   La réplication stable et constitutive

L'initiation d'une nouvelle ronde de réplication du chromosome de *E. coli* requiert une synthèse de protéine *de novo* (Lark, 1972). Cependant, certains mutants ont été isolés par leur propriété à initier la réplication en absence de nouvelle synthèse protéique (Kogoma, 1978). En raison de leur phénotype, ils ont été nommé mutant de réplication stable et constitutive (cSDR, « constitutive and stable DNA replication »). Ces mutants peuvent même tolérer l'inactivation du gène *dnaA* ou la délétion de la région *oriC* du chromosome (Kogoma *et al.*, 1985). La mutation responsable de ce phénotype a été localisée dans le gène *rnhA*, dont le produit muté ne démontre pratiquement pas d'activité (Ogawa *et al.*, 1984). La RNase H agit donc comme facteur de spécificité afin que l'initiation de la réplication ne puisse s'effectuer qu'à l'origine de réplication *oriC*. D'autres facteurs empêcheraient aussi l'activation de la réplication ailleurs qu'à *oriC* sur le chromosome : la topoisomérase I (Kaguni and Kornberg, 1984) et la protéine HU (Dixon and Kornberg, 1984) auraient un rôle important dans le maintient de la spécificité de réplication a *oriC*. La topoisomérase I agirait en déstabilisant les amorces situées ailleurs qu'à *oriC*, qui eux, seraient stabilisée par la protéine DnaA. De plus, certaines mutations de *dnaA* peuvent être compensées par une délétion du gène *topA* (Louarn *et al.*, 1984). Cependant, contrairement aux mutations *rnhA*, la protéine DnaA ainsi que *oriC* sont toujours essentielles en absence de topoisomérase I.

La réplication stable et constitutive est initiée aux sites appelés *oriK* (deMassy *et al.*, 1984). Plusieurs de ces sites existent sur le chromosome sans qu'aucun ne semble être favorisé. De plus, la synthèse protéique *de novo* n'est pas essentielle pour les activer. Toutefois, la transcription demeure critique et la présence de la protéine RecA est requise (Kogoma *et al.*, 1985). Le modèle de réplication aux origines *oriK* propose que la transcription à partir de promoteurs spécifiques permettrait la formation d'hybrides ARN-ADN stabilisés par l'absence de RNase H. Ces hybrides constitueraient ainsi des endroits propices à l'initiation de la réplication.

6.4.4   Origine de réplication de l'ADN mitochondrial

Chez les vertébrés, l'ADN de mitochondrie code pour 13 protéines faisant partie du complexe d'enzymes de la chaîne respiratoire qui en compte au total une centaine. Cet ADN contribue aussi à la synthèse de 2 ARNr et de 22 ARNt requis pour la traduction des ARNm de mitochondrie. Le contenu génétique des mitochondries est extrêmement bien conservé d'une espèce à l'autre. Cependant, les éléments impliqués dans la réplication varient considérablement (Shadel and Clayton, 1997).

Le chromosome de mitochondrie se retrouve sous forme circulaire et surenroulée et sa taille varie de 16 à 18 kb. Les deux brins ADNmt qui le constitue, peuvent être facilement séparés par un gradient dénaturant grâce à une différence notable du contenu en G et T de ces brins, appelés « heavy » et « light ». La transcription sur chacun de ces brins débute à partir d'un seul promoteur respectif qui produit un long ARN polycistronique éventuellement clivé afin de générer les ARNm, ARNr et ARNt. Le brin « light » porte le promoteur LSP (« light-strand promoter ») et le brin « heavy » contient le HSP (« heavy-strand promoter »).

Le chromosome de mitochondrie contient une région non codante juste après le promoteur LSP représentant l'origine de réplication du brin « heavy » (O_H). Cette région ainsi que la section portant le promoteur s'appelle D-loop (displacemement-loop). Une amorce ADN, découlant de l'arrêt de la réplication du brin heavy, forme la structure D-loop stable. Celle-ci est responsable de la régulation de la réplication du

chromosome de la mitochondrie. Le rôle précis du D-loop demeure plus ou moins précis. Il pourrait permettre de garder un nombre suffisant de chromosomes prêts à amorcer la réplication, réguler la transcription, ou permettre la ségrégation des molécules (Shadel and Clayton, 1997).

Plusieurs évidences expérimentales ont démontrées que l'ARN provenant de la transcription du LSP pouvait servir d'amorce de réplication du brin heavy (Clayton, 1991). On retrouve dans la région D-loop, trois « conserved sequence blocks » (CSBI, CSBII et CSBIII) semblables chez les humains, les souris et les rats. La transcription *in vitro* d'une région CSB, riche en G et C, provoque la formation d'un R-loop persistant (Xu and Clayton, 1995). Une fois l'hybride formé, la RNase MRP (« mitochondrial RNA processing endoribonuclease ») coupe dans l'ARN afin de générer une extrémité 3' OH. La polymérase ADN peut alors amorcer la réplication. La formation de R-loop à l'origine de réplication de mitochondrie montre une sensibilité à la RNase H et à la RNase A (Xu and Clayton, 1995). Les auteurs expliquent ce résultat par la présence d'un équilibre dynamique entre l'ARN libre et hybridé à la matrice.

Les transcrits ARN formant le R-loop dans les origines de réplication mitochondriales et ColE1 partagent une séquence nucléotidique riche en guanines qui est importante pour la formation d'un hybride persistant. Ceci supporte l'idée que les interactions rG-dC sont primordiales dans la stabilisation des hybrides ARN-ADN.

6.5   *L'effet de l'élongation de la transcription sur la recombinaison*

Chez la levure *S. cerevisiae*, la transcription stimule la recombinaison durant la division mitotique (Stewart and Roeder, 1989; Thomas and Rothstein, 1989). On observe aussi une recombinaison accrue, dépendante de la transcription, dans les séquences répétées en tandem situées en aval des promoteurs spécifiques aux polymérases I et II (Keil and Roeder, 1984; Voelkel-Meiman *et al.*, 1987; Stewart and Roeder, 1989; Thomas and Rothstein, 1989). Chez les cellules de mammifères, la transcription à partir d'un promoteur de virus causant une tumeur de glande mammaire stimule la recombinaison (Nickoloff, 1992).

Le mécanisme de la commutation (« switching ») de classe des immunoglobulines requiert la recombinaison entre les régions de commutation dans l'extrémité 3' hypervariable des gènes. Cette recombinaison est essentielle dans la production d'anticorps par les lymphocytes B. Il a été observé qu'une forte transcription se produit juste avant la recombinaison de cette région. La transcription *in vitro* de la région de commutation mène à la formation d'un hybride ARN-ADN (Reaban and Griffin, 1990; Reaban *et al.*, 1994). De plus, la formation de l'hybride ne se produit que lorsque la transcription se fait dans l'orientation physiologique autant *in vitro* que *in vivo* (Daniels and Lieber,1995ab). Certaines régions de commutation peuvent former un hybride indépendamment de la topologie de l'ADN, alors que d'autres nécessitent du surenroulement négatif (Reaban *et al.*, 1994).

Chez la levure *S. cerevisiae*, les quelques 200 copies des opérons ribosomaux, mesurant chacun 9 kb, sont placés en tandem sur le chromosome XII. On observe que dans un double mutant *top1 top2*, la recombinaison dans ces opérons augmente de 50 à 200 fois au moment de la mitose (Christman *et al.*, 1988). Toujours chez ce même mutant, il a été démontré que la moitié de l'ADNr est excisé en cercles extrachromosomaux portant chacun un ou plusieurs opérons de 9 kb (Kim and Wang, 1989). Les auteurs ont aussi dénoté que l'expression sur un plasmide des gènes *top1* et *top2* permet la réintégration des cercles dans le chromosome.

Le lien entre la recombinaison et les topoisomérases semble aussi important chez les bactéries. Des résultats provenant de notre laboratoire démontrent que chez les mutants *topA gyrB*(Ts), le taux de recombinaison entre les opérons ribosomaux augmente de 20 à 50 fois comparé au témoin *gyrB*(Ts) (E. Massé et M. Drolet, résultats non publiés).

7 Articles publiés

7.1 « *DNA topoisomerases regulates R-loop formation during transcription of the* rrnB *operon in* Escherichia coli » *par Massé* **et al.,** *(1997).*

Plusieurs études *in vitro* et *in vivo* ont suggéré que les topoisomérases ADN ont un rôle important à jouer dans la régulation de la formation de R-loops durant la transcription chez *Escherichia coli*. Les travaux présentés ici montrent plusieurs évidences génétiques et biochimiques qui suggèrent fortement la formation de ces structures durant la transcription d'une portion de l'opéron ribosomal *rrnB*. De plus, l'activité des topoisomérases régule la formation des R-loops. Nous avons démontré qu'un plasmide multicopie (pBR322) portant une portion fortement transcrite de l'opéron *rrnB* ne peut être introduit dans une souche *topA*, à moins que la RNase H ne soit surproduite. La transcription du fragment *Hind*III (567 pb) provenant de l'opéron *rrnB* permet l'extraction de plasmides portants une quantité significative de R-loops (conformères), et ce, de manière dépendante de l'absence du gène *topA* et de la quantité intracellulaire de RNase H. Si la gyrase devient suffisamment active, on observe la formation de plasmide hypernégativement surenroulé lors de la transcription du même fragment dans un mutant *topA*. La formation de ce type de topoisomères reflète la présence de R-loops puisqu'ils sont sensibles à la concentration intracellulaire de RNase H. Enfin, des expériences *in vitro* avec des polymérases ARN phagiques ont montrées que la transcription du fragment *Hind*III, sur une matrice surenroulée, provoque la formation de R-loops.

DNA Topoisomerases Regulate R-loop Formation during Transcription of the *rrnB* Operon in *Escherichia coli**

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Recent *in vivo* and *in vitro* studies have suggested an important role for DNA topoisomerases in regulating R-loop formation during transcription in *Escherichia coli*. In the present report we present genetic and biochemical evidence strongly suggesting that R-loop formation can occur during transcription of a portion of the *rrnB* operon and that it is regulated by DNA topoisomerase activity. We found that a multicopy plasmid (pBR322) carrying an heavily transcribed portion of the *rrnB* operon cannot be transformed in *topA* mutants unless RNase H is overproduced. Transcription of the 567-base pair *Hind*III fragment from the *rrnB* operon allows the extraction of large amount of R-looped plasmid DNAs from a *topA* mutant, in a manner that depends on the intracellular level of RNase H activity. When DNA gyrase is sufficiently active, hypernegatively supercoiled plasmid DNA is produced if the same DNA fragment is transcribed in a *topA* mutant. The formation of such topoisomers most likely reflect the presence of extensive R-loops since it is sensitive to the intracellular level of RNase H activity. Finally, the formation of R-looped plasmid DNAs in an *in vitro* transcription system using phage RNA polymerases is also detected when the 567-base pair *Hind*III fragment is transcribed on a negatively supercoiled DNA template.

During the process of transcription, the nascent RNA molecule is normally displaced from the DNA template strand to be translated (mRNAs) or to participate in the process of translation (stable RNAs: tRNAs and rRNAs). Indeed, early results have suggested that *Escherichia coli* RNA polymerase has a function allowing proper RNA displacement from the template strand (1). However, in some cases the RNA has to remain associated with the template strand to serve as a primer for DNA replication initiation. The best studied example in this regard is the replication origin of ColE1 plasmid DNA (2, 3). In addition, several observations suggest that R-loop formation can occur in a rather nonspecific manner if DNA gyrase, the enzyme responsible for the introduction of negative supercoiling into the DNA of bacteria, is present during transcription. In *in vitro* replication systems for *oriC* containing plasmid DNAs and ColE1 plasmid derivatives and where RNA polymerase and DNA gyrase are present, RNase H and *E. coli* topoisomerase I, an enzyme that specifically relaxes negatively super-

coiled DNA, are required to maintain the specificity in the process of initiation at *oriC* or at the ColE1 origin of replication, respectively (4–6). This result is best explained by the introduction of negative supercoiling by DNA gyrase providing the driving force for R-loop formation at sites other than the normal origins of replication. The annealed RNAs can be used as primers to initiate replication at these sites. RNase H, an endoribonuclease degrading the RNA moiety of an RNA-DNA hybrid, eliminates these primers, whereas *E. coli* topoisomerase I by counteracting DNA gyrase activity, prevents their formation or destabilizes them. Recent *in vitro* experiments have shown that the production of hypernegatively supercoiled DNA during transcription in the presence of DNA gyrase was due to R-loop formation (7, 8). Many plasmid DNAs tested in this system were shown to become hypernegatively supercoiled during transcription in the presence of DNA gyrase but in the absence of RNase H, suggesting that extensive R-loop formation was a rather general phenomenon in the presence of DNA gyrase (7, 8). Moreover, *E. coli* DNA topoisomerase I was shown to efficiently abolish the formation of such supercoiled DNA during transcription, and hence R-looping as well. A model for the regulation of R-loop formation was therefore proposed in which DNA gyrase, with its supercoiling activity, favors the formation of R-loops, while DNA topoisomerase I, by relaxing negative supercoiling, inhibits the formation of such structures. In support of this model, it has been recently demonstrated that an R-loop can be a hot spot for DNA relaxation by *E. coli* DNA topoisomerase I (8).

To study the putative role of DNA topoisomerase I in the regulation of R-loop formation *in vivo*, it was necessary to construct new *topA* mutants. The reason is that to survive, *topA* mutants need to acquire compensatory mutations that most often lie in a *gyr* gene and as a result, lower DNA gyrase activity. Moreover, some of these strains such as the widely used DM800 with the *gyrB225* mutation, has one or more additional compensatory mutation(s) (9). Since these mutations arise to compensate for the absence of DNA topoisomerase I, they will most likely mask some of the effects associated with the loss of *topA* gene function. Various *topA* mutants with a conditional compensatory phenotype were thus constructed (10). These strains have a *gyrB*(Ts) mutation and as a result, they can grow at 37 °C because gyrase activity is then low enough to compensate for the loss of *topA*. At 30 °C, they basically do not grow because gyrase regains a more wild-type level of activity that can no longer compensate for the absence of DNA topoisomerase I. Such strains are therefore cold-sensitive and are also temperature-sensitive (Ts) because of the presence of the *gyrB*(Ts) allele. By exposing these strains to the non-permissive cold temperature it is thus possible to study the true effects of loosing *topA* on cell physiology. Since RNase H overproduction allowed these mutants to grow at low temperature, it was concluded that R-loop formation was a major

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TABLE I

E. coli strains used in this study

Strains	Genotype	Reference
RFM443[a]	*rpsL galK2, Δlac74*	10
RFM445[a]	*rpsL galK2, Δlac74, gyrB221*(cou^R), *gyrB203*(Ts)	10
RFM475[a]	*rpsL galK2, Δlac74, gyrB221*(cou^R), *gyrB203*(Ts) *Δ(topA cysB)204*	10
RFM480[a]	*rpsL galK2, Δlac74, gyrB221*(cou^R), *gyrB203*(Ts) *topA20::Tn10*	10
DM800	*Δ(topA cysB)204 gyrB225, acrA13*	9
EM205	DM800 *topA+, cysB+*	This work
EM209	DM800 *topA+, cysB+, gyrB+*	This work

[a] These strains are all derived from N99 (*rpsL galK2*).

problem for the cell in the absence of DNA topoisomerase I. Moreover, a combination of *topA* and *rnhA* (encoding for RNase HI) mutations was found to be non-viable and the *gyrB*(Ts) mutation was shown to correct some phenotypes associated with an *rnhA* mutation at 37 °C. It was thus suggested that *gyr* mutations arise in *topA* mutants to reduce R-loop formation (10). These *in vivo* data are therefore in perfect agreement with the *in vitro* results and suggest an important role for DNA topoisomerases, and hence DNA supercoiling, in the regulation of R-loop formation. In view of the important roles of these enzymes in the regulation of DNA functions, it is thus interesting to consider the R-loop structure as a putative regulator of various genomic activities. However, this model needs to be supported by data showing that R-loop formation indeed occurs during transcription of certain genes in *E. coli* and that it is regulated by DNA topoisomerase activity. In the present report we present genetic and biochemical evidence that R-loop formation can occur during transcription of an *rrn* operon both *in vivo* and *in vitro* and that it is influenced by DNA topoisomerase activity.

EXPERIMENTAL PROCEDURES

E. coli Strains—E. coli strains used are listed and described in Table I. The detailed protocol used to construct the DM800 derivatives by P1*vir* transduction will be presented elsewhere. The RFM480 cold-sensitive strain is well described in the Introduction and in Ref. 10.

Plasmids—pNO1302 is a pBR322 derivative containing a non-functional *rrnB* operon with its complete regulatory region including P1 and P2 promoters (11; Fig. 1). It was constructed by removing the 2451- and 53-bp[1] adjacent *Sal*I fragments of the *rrnB* operon of pNO1301. This deletion removed the 3' end of the 16 S rRNA gene, the spacer region, and the 5' end of the 23 S rRNA gene. pEM501 was obtained by deleting the 451-bp *Bst*BI-*Stu*I fragment of pNO1302 (nucleotides 1113 to 1563 according to Ref. 12) to remove the regulatory region of *rrnB* including both promoters. pEM520 was constructed by deleting the 567- and 3180-bp *Hin*dIII DNA fragments of pNO1302, removing all the *rrnB* coding sequence downstream of the first *Hin*dIII site of the *rrnB* operon, and the 346-bp *Bam*HI-*Hin*dIII fragment of pBR322. pTrc99a, obtained from Pharmacia, is an expression vector derived from pBR322 that carries a multiple cloning site downstream of a Shine-Dalgarno sequence and the IPTG-inducible P_{trc} promoter. This vector also carries the *lac* I^q gene allowing good transcriptional repression of P*trc*, in the absence of IPTG. pMD210 is a derivative of pTrc 99a in which the Shine-Dalgarno sequence has been deleted by *Bal*31 nuclease digestion from *Nco*I ends, as described (13). This deletion removed nucleotides 251 to 289 of the pTrc 99a vector. pMD217 (Fig. 2) was constructed by inserting a synthetic *boxA* sequence (14) with *Sal*I (5') and *Hin*dIII (3') cohesive ends into the same sites of pMD210. This synthetic *boxA* sequence was obtained by annealing to one another the two oligonucleotides with the following nucleotide sequence: 5'-TCGACACT-GCTCTTTAACAATTTA and 5'-AGCTTAAATTGTTAAAGAGCAGTG. pMD306 and pMD308 are derivatives of pMD217 in which the 567-bp *Hin*dIII fragment from the *rrnB* operon on pNO1302 was inserted into the *Hin*dIII site (nucleotides 1598 to 2165 according to Ref. 12), respectively, in its original and its reverse orientation relative to P*trc* (Fig. 2).

A region from pMD306 and pMD308 including the 5' end of the *Hin*dIII *rrnB* fragment, the *boxA* element, and the P*trc* promoter was sequenced and was found to be as expected (data not shown). pEM001 and pEM003 were constructed by first subcloning the *Sal*I-*Eco*RI fragment respectively from pSK760 (15) carrying the *rnhA* gene and pSK762c (16) carrying a mutated *rnhA* gene, into the same sites of the pBluescript KS vector (from Stratagene). The *Xba* I-*Hin*cII fragment from the respective resulting plasmid was then subcloned within the same sites of pACYC184 to give pEM001 and pEM003. pJP459 and pJP461 were obtained by cloning the 567-bp *Hin*dIII fragment of *rrnB* into the same site of the pBluescript KS vector, respectively, in the reverse and the physiological orientation relative to the vector map numbering.

Media and Growth Conditions—Unless otherwise indicated, bacteria were grown in LB media at the temperature indicated in the table and figure legends. When needed, antibiotics were added as follows: ampicillin at 50 μg/ml, and chloramphenicol at 30 μg/ml. Because of the *acrA13* mutation in the DM800 derivatives that renders these cells more permeable to many antibiotics (9), chloramphenicol was used at 10 μg/ml, for these cells. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-glucoside were obtained from Sigma.

Molecular Biology Techniques—The molecular biology techniques were performed essentially as described (13). CaCl$_2$ transformations were done as described in Drolet *et al.* (10).

Electrophoresis in the Presence of Chloroquine—One-dimensional and two-dimensional agarose gel electrophoresis in the presence of chloroquine were performed essentially as described (7), except that $0.5 \times$ TBE (13) was used instead of $0.5 \times$ TPE. Chloroquine was used at the concentration indicated in the figures. After electrophoresis, agarose gels from one-dimensional electrophoresis were stained with ethidium bromide and photographed under UV light. Agarose gels from two-dimensional electrophoresis were dried and prepared for *in situ* Southern hybridization as described (17).

Extraction of R-looped Plasmid DNAs—To examine if plasmid DNAs extracted from various strains contained R-loops, a clear lysate protocol was used (18). Bacterial cells carrying pMD306 or pMD308 DNAs were grown at the indicated temperature to an OD$_{600}$ of 0.4 at which time they were split evenly (10 ml) into two tubes. IPTG at 1 mM final was then added to one tube and both tubes were incubated for an additional 75 min. Cells were transferred in a tube half full of ice and pelleted and resuspended in 200 μl of an ice-cold solution containing 50 mM Tris (pH 8.0) and 25% sucrose. Forty μl of an ice-cold solution made of 0.25 M Tris (pH 8.0) and lysozyme at 5 mg/ml were added and the cells were incubated on ice for 5 min. After the addition of 80 μl of ice-cold 0.25 M EDTA (pH 8.0) and a 5 min incubation on ice, 320 μl of 2% Triton X-100 in water were added. The sample was incubated on ice for 10 min with periodic gentle agitation and then centrifuged for 30 min at 4 °C in a microcentrifuge. The globular pellet (containing chromosomal DNA and cell debris) was removed and SDS at 0.5% and proteinase K at 0.5 mg/ml were added to the supernatant. After a 30-min incubation at 37 °C, the sample was extracted once with phenol, once with chloroform, and the nucleic acids were precipitated with ethanol. The DNA pellet was resuspended in 100 μl of TE buffer (13) and the sample was extracted once with phenol and twice with chloroform before being precipitated with ethanol and resuspended in 40 μl of TE buffer. Two aliquots of 10 μl were treated with 250 ng of RNase A in a solution made of 10 mM Tris (pH 8.0), 20 mM MgCl$_2$, and 100 mM NaCl. Twenty ng of *E. coli* RNase HI (from Robert J. Crouch, National Institutes of Health) were added to one tube and both aliquots were incubated at 37 °C for 1 h. After one phenol extraction, the samples were analyzed by agarose gel electrophoresis (1%) in $1 \times$ TAE (13).

In Vitro Transcription Reactions— In vitro transcription reactions for the detection of R-loop formation were performed with T3 and T7 RNA polymerases (6 units) purchased from Stratagene and Life Technologies, Inc. A 25-μl reaction mixture containing 40 mM Tris-HCl (pH 8.0), 8 mM MgCl$_2$, 25 mM NaCl, 2 mM spermidine, 30 mM dithiothreitol, 400 μM each of NTP, and 0.33 μg of plasmid DNA with a native superhelical density, was prepared in a tube on ice. When indicated, 5 ng of RNase H and/or 1 μg of RNase A were added during transcription. After a 20-min incubation at 37 °C, the reaction was stopped by 0.2% SDS final and extracted with a mixture of phenol-chloroform. The samples were then incubated at 37 °C for 1 h in the presence of 250 ng of RNase A and, when indicated, 20 ng of RNase H. The samples were analyzed by agarose gel electrophoresis (1%) in $1 \times$ TBE (13).

RESULTS

Transcription of a Non-functional rrnB Operon on a Multicopy Plasmid Is a Major Problem for topA Mutants That do Not

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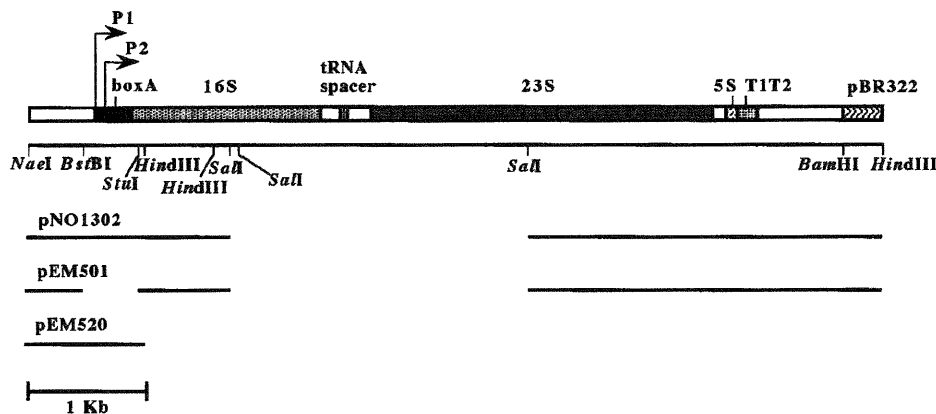[1] The abbreviations used are: bp, base pair(s); IPTG, isopropyl-1-thio-β-D-galactopyranoside.

FIG. 1. **A schematic drawing of the plasmid constructions carrying various portions of the *rrnB* operon and used in the transformation experiments.** The entire *rrnB* operon is included within a *NaeI-BamHI* DNA fragment inserted into the same sites of pBR322 to give pNO1301 (11). pNO1302 (11) was derived from pNO1301, and pEM501 and pEM520 (this study) were derived from pNO1302 as described under "Experimental Procedures." The DNA segment containing the 5'-regulatory elements including the promoter regions and the *boxA* sequence for antitermination is shown in *black*. *White* area represent DNA sequences that do not encode for stable RNAs. 16 S, 23 S, tRNA spacer, and 5 S, represent regions encoding for the corresponding stable RNAs. *T1 T2* indicates the tandem transcription terminators of the *rrnB* operon.

Overproduce RNase H—Our *in vivo* results strongly suggested that R-loop formation was a major problem in the absence of DNA topoisomerase I but did not indicate anything relating to the specificity, the frequency, or the consequences of R-loop formation (10). For several reasons we considered that R-loop formation might occur during transcription of the ribosomal RNA genes (*rrn* operons). First, since they are heavily transcribed (they contribute to more than half of the transcriptional activity in an exponentially growing culture of *E. coli*); they are statistically more prone to R-loop formation. Second, since rRNAs are not translated they are free to hybridize with the template strand, as opposed to mRNAs that are bound by ribosomes. Third, we observed that the growth of our cold-sensitive *topA* mutants was very sensitive to nutritional shift-ups unless RNase H was overproduced (10). Sensitivity of *rnhA* mutants to nutritional shift-ups has also been reported (19). Under nutritional shift-up conditions, the cells have to face a sudden demand for a large amount of ribosomes and therefore, for rRNAs at the very beginning. A recent report demonstrates that the seven chromosomal copies of the *rrn* operon confer growth advantage over cells that have two *rrn* operons deleted, if cells are exposed to nutritional shift-ups (20). These results suggest that it is during nutritional shift-ups that the *rrn* operons experiment the maximum rate of transcription and are thus more prone to R-loop formation. Fourth, we found that double *nusB5-topA* and *nusB5-rnhA* mutants are barely viable.[2] One of the reported phenotype of the *nusB5* mutant is a partial inhibition of *rrn* operons transcription elongation (21). However, these mutants are still able to make the same number of ribosomes as wild-type cells, apparently by increasing the frequency of *rrn* operon transcription initiation, in agreement with the feedback regulation model of ribosome synthesis (11).

We reasoned that, if R-loop formation does occur during rRNA synthesis, increasing the total amount of cellular *rrn* transcription by introducing a multicopy plasmid carrying an *rrn* operon, it could result in growth inhibition of *topA* mutants, because of a possible depletion of the negative regulators of R-loop formation (*e.g.* RNase H). In the beginning we used the plasmid pNO1302 (Fig. 1), a derivative of the multicopy plasmid pBR322, that was previously used in experiments to support the feedback regulation model of ribosome synthesis (11). pNO1302 contains a deleted *rrnB* operon with an intact pro-

moter region but a *SalI* deletion removing the 3' end of the 16 S rRNA gene, the spacer region, and the 5' end of the 23 S rRNA gene (11). Since the *rrnB* operon carried by this multicopy plasmid is non-functional, it will not be subject to feedback regulation and, as a result, a cell carrying this plasmid will produce much more untranslated RNAs.

We thus transformed this pBR322 derivative in various bacterial strains. As can be seen in Table II, pNO1302 can transform all *topA+* strains whether they overproduce RNase H (pEM001) or not (pEM003). However, pNO1302 could only be transformed in *topA* null mutants providing that RNase H was overproduced. This was true for all *topA* mutants tested, including the widely used DM800 strain (Table II). The fact that a pNO1302 derivative with a deletion removing the two *rrnB* promoters (pEM501) is able to transform *topA* null mutants irrespective of the cellular amount of RNase H suggests that this is due to heavy transcription from these very strong *rrnB* promoters. Moreover, the fact that a pNO1302 derivative carrying both *rrnB* promoters but in which almost all the *rrnB* coding sequence is deleted (pEM520) is able to transform *topA* mutants not overproducing RNase H, indicates that the problem is related to transcription of the *rrnB* operon. We also analyzed, by restriction enzyme digestions and electrophoresis, the plasmid DNA extracted from several RFM480 cells (*topA::Tn10*) transformed with pNO1302. Although intact pNO1302 was always found from RFM480 cells overproducing RNase H, the very few RFM480 transformants not overproducing RNase H were found to contain low-molecular weight pNO1302 derivatives, in which almost the entire *rrnB* operon was deleted (data not shown).

Transcription of the 567-bp rrnB HindIII Fragment Allows the Extraction of R-looped Plasmid DNA from a topA Mutant—Our results suggested that R-loop formation was taking place during transcription of the *rrnB* operon on pNO1302, but did not indicate if specific *rrnB* sequences were involved. In addition, since the evidence we obtained supporting R-loop formation was based on growth phenotypes and therefore being rather indirect, we decided to address this question in a more direct manner by looking at the presence of R-loops on our plasmid constructs, extracted from the cold-sensitive *topA* mutant. The assay is based on the fact that the presence of R-loops on plasmids will alter their migration in an agarose gel, mainly due to DNA relaxation, since DNA unwinding must occur to allow RNA-DNA hybrid formation. This DNA relaxation caused by the presence of R-loops will produce conformers, not

For the preparation of competent cells, the bacteria were grown in TB medium as described (10). Incubations at 37 °C on LB media with the required antibiotics were for 40 h and for 60 h for RFM480 cells containing pEM003. +: indicates more than 500 transformants. −: indicates less than 15 transformants.

Bacterial strains	pBR322 derivatives		
	pNO1302	pEM501	pEM520
RFM443/pEM001	+	+	+
RFM443/pEM003	+	+	+
RFM445/pEM001 [gyrB(Ts)]	+	+	+
RFM445/pEM003 [gyrB(Ts)]	+	+	+
RFM475/pEM001 [ΔtopA, gyrB(Ts)]	+	+	+
RFM475/pEM003 [ΔtopA, gyrB(Ts)]	−	+	+
RFM480/pEM001 [topA::Tn10, gyrB(Ts)]	+	+	+
RFM480/pEM003 [topA::Tn10, gyrB(Ts)]	−	+	+
DM800/pEM001 [ΔtopA, gyrB225]	+	+	+
DM800/pEM003 [ΔtopA, gyrB225]	−	+	+
EM205/pEM001 [gyrB225]	+	+	+
EM205/pEM003 [gyrB225]	+	+	+
EM209/pEM001	+	+	+
EM209/pEM003	+	+	+

topoisomers, since the DNA linking number is unchanged. Disappearance of the conformers upon RNase H treatment will be taken as a strong evidence for the presence of R-loops.

We thus designed a system allowing the subcloning of *rrnB* DNA fragments under the control of the inducible P*trc* promoter. In this vector, the Shine-Dalgarno sequence following the inducible promoter has been deleted, but a synthetic *boxA* sequence has been added (Fig. 2, pMD217). Because our preliminary results indicated that the NusB protein that acts in the process of antitermination by interacting with a *boxA* element (reviewed in Ref. 22) seems to have an effect on R-loop formation,[3] we considered the possibility that a *boxA* sequence could be essential for the formation of such structures. The strong ρ-independent *rrnB* T1 T2 terminator sequence is also present in this vector, in such a way that transcription from P*trc* will be terminated whether or not a *boxA* sequence is present. In addition, since transcription from P*trc* on these vectors is tightly regulated owing to the presence of the *lacI*q gene, it is therefore possible to clone *rrnB* sequences and to introduce the resulting plasmid DNAs in our *topA* mutants. Our work first began with the subcloning of the 567-bp *Hind*III DNA fragment from the *16 S* gene of the *rrnB* operon, previously used to study the antitermination system of *rrn* operons (23, 24). This *Hind*III fragment was thus subcloned in both orientations into the *Hind*III site of pMD217 to produce pMD306 and pMD308, respectively, carrying this *Hind*III fragment in the original (or physiological) and the reverse orientation, relative to the IPTG-inducible P*trc* promoter (Fig. 2). In the beginning we observed that the growth on LB plates of our *topA* mutants not overproducing RNase H and carrying pMD306 or pMD308 was inhibited upon IPTG induction and when a selective pressure was kept for the maintenance of the plasmid DNAs (+ampicillin). We also observed that this growth inhibition was more severe for *topA* mutants carrying pMD306 as compared with the same mutants carrying pMD308. These observations suggested to us that R-loop formation was occurring during transcription of this *Hind*III fragment, mainly in its original orientation, and that such R-loops were interfering with the maintenance of the plasmid DNAs.

Plasmid DNAs were extracted as described under "Experimental Procedures," from RFM480 carrying either pMD306 or
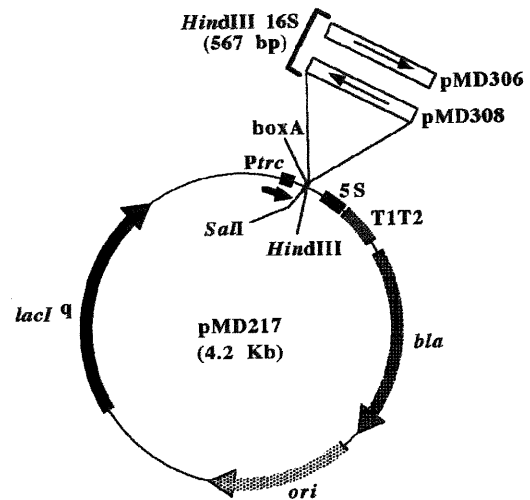

FIG. 2. **A schematic drawing of the plasmid constructions used to study R-loop formation in the** *topA*::Tn*10* **RFM480 strain.** The *arrows* indicate the physiological orientation of the various elements in the plasmid constructs. The DNA fragment containing the *rrnB* transcription terminators (T1T2) also include a portion of the 5 S rRNA (*5S*). A more detailed description of these plasmid constructs is presented under "Experimental Procedures."

pMD308 and grown in the presence or not of 1 mM IPTG at 35 °C. As can be seen in Fig. 3, a large amount of conformers that disappear upon RNase H treatment (compare *lane 1* with *lane 2*) is only visible for pMD306 extracted from cells grown in the presence of 1 mM IPTG (compare *lane 1* with *lane 5*). We can also observe that the migration of almost all plasmid DNAs is retarded suggesting that nearly 100% of these pMD306 DNAs contains R-loops (*lane 1*). Basically no conformers are observed for pMD308 extracted from cells grown in the presence of 1 mM IPTG (compare *lane 3*, pMD308, with *lane 1*, pMD306). These results show that under our experimental conditions, a significant amount of R-loops is only detected when the *rrnB Hind*III fragment is transcribed (IPTG induction) in its physiological orientation (pMD306).

Early results have shown that R-loop formation could be induced by the use of protein denaturants such as SDS and phenol, when a negatively supercoiled DNA template was used in *in vitro* transcription experiments (1). Although we considered unlikely this possibility especially because the formation of conformers is dependent on the DNA fragment orientation (pMD306 *versus* pMD308) and also because the detrimental effect of heavily transcribed *rrnB* sequences on the growth of *topA* mutants is corrected by RNase H overproduction, we performed a simple experiment to address this question. One prediction that could be made if R-loop formation was an artifact of extraction is that RNase H overproduction *in vivo* should not have any effect on the formation of conformers. On the contrary, if R-loop formation really occurs *in vivo*, RNase H overproduction should abolish or decrease the production of such conformers. The results presented in Fig. 4 clearly show that RNase H overproduction *in vivo* strongly decreased the production of conformers and hence R-loop formation (compare *lane 1*, RNase H overproduction with *lane 3*, no RNase H overproduction). This result demonstrates that at least the formation of a large fraction of the conformers is due to R-loop formation *in vivo*. A net decrease in the formation of conformers is also observed in an *in vitro* transcription system when the same DNA fragment is transcribed in the presence of RNase H (see below).

Transcription of the 567-bp rrnB HindIII Fragment Allows the Extraction of Hypernegatively Supercoiled Plasmid DNA

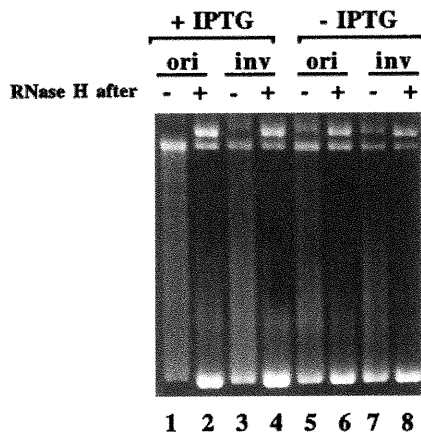[3] E. Massé and M. Drolet, unpublished data.

FIG. 3. **Extraction of R-looped plasmid DNAs from the** *topA*::Tn*10* **RFM480 strain in function of the orientation of the 567-bp** *Hind*III *rrnB* **fragment.** Cells were grown at 35 °C as described under "Experimental Procedures" in the presence or absence of 1 mM IPTG as indicated. Plasmid DNAs, either pMD306 or pMD308, were extracted as described under "Experimental Procedures." *ori* and *inv*, indicate, respectively, the physiological orientation (pMD306) and the reverse orientation (pMD308) of the 567-bp *rrnB Hind*III fragment relative to the P*trc* promoter. *RNase H after* indicates that RNase H was added (+) or not (−) after the extraction of the plasmid DNAs and therefore, before electrophoresis.

from a topA Mutant When DNA Gyrase Is Sufficiently Active—Previous *in vitro* studies have shown that transcription in the presence of DNA gyrase can lead to extensive R-loop formation and the subsequent generation of hypernegatively supercoiled plasmid DNAs upon R-loops removal (7, 8). In one of this study, transcription of the 567-bp *rrnB Hind*III fragment with phage RNA polymerases and in the presence of DNA gyrase, was shown to generate such hypernegatively supercoiled DNA and hence very long R-loops (8). We can therefore predict that extensive R-loop formation during transcription of this *rrnB Hind*III fragment in a *topA* mutant should generate hypernegatively supercoiled DNA, if DNA gyrase is sufficiently active. Our initial experiments failed to detect a significant amount of such topoisomers when pMD306 DNA was extracted from our cold-sensitive *topA* mutant grown at 35 °C, following IPTG induction (data not shown). This is most likely due to the fact that DNA gyrase, owing to the *gyrB*(Ts) allele, is too weak at 35 °C to promote extensive R-loop elongation. We therefore performed an experiment in which the *topA* mutant cells were grown at 35 °C before being incubated at 30 °C for 75 min, to activate DNA gyrase. Plasmid DNAs, pMD306 or pMD308, were then extracted as described under "Experimental Procedures." The result shown in Fig. 5*A* suggest that extensive R-loop formation occurred when the 567-bp *Hind*III fragment was transcribed (+IPTG) in its physiological orientation, since the migration of almost 100% of the extracted pMD306 DNAs was retarded during electrophoresis (compare *lane 1*, no RNase H, with *lane 2*, RNase H). Significant R-loop formation also occurred when the *rrnB Hind*III fragment was transcribed (+IPTG) in its reverse orientation since the migration of a large proportion of the extracted pMD308 DNAs was retarded during electrophoresis (compare *lane 3*, no RNase H, with *lane 4*, RNase H). However, it is also obvious that under our experimental conditions, a larger amount of R-loops is detected when the *rrnB Hind*III fragment is transcribed in its physiological orientation (compare *lane 1*, pMD306 with *lane 3*, pMD308).

We next loaded an aliquot of the samples from the experiments shown in Fig. 5*A* on an agarose gel with chloroquine, to detect the presence of hypernegatively supercoiled plasmid
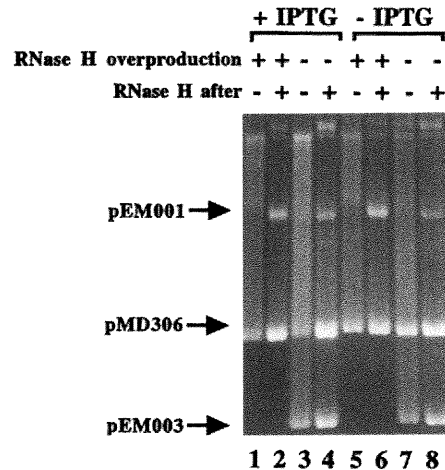

FIG. 4. **Effect of RNase H overproduction in the** *topA*::Tn*10* **RFM480 strain on the formation of R-looped pMD306 DNAs.** Cells were grown at 35 °C as described under "Experimental Procedures" in the presence or absence of 1 mM IPTG as indicated. pMD306 DNA was extracted as described under "Experimental Procedures." *RNase H overproduction* indicates that RNase H was overproduced *in vivo* (+) due to the presence of pEM001 or that RNase H was not overproduced (−) due to the presence of pEM003. *RNase H after* indicates that RNase H was added (+) or not (−) after the extraction of the plasmid DNAs and therefore, before electrophoresis. Note that pEM001 is always isolated as a dimeric form, whereas pEM003 is mostly found as a monomeric form. The reason for that is unknown.

DNAs. Before loading on such gel, the DNA samples were treated with RNase H to reveal the supercoiling level after electrophoresis and ethidium bromide staining. As can be seen in Fig. 5*B* hypernegatively supercoiled plasmid DNA is produced following transcription of the 567-bp *rrnB Hind*III fragment in both orientations (*lanes 1* and *2*, [- -]). Despite the fact that more R-loops are detected on pMD306 than pMD308 (Fig. 5*A* compare *lane 1* with *lane 3*), roughly the same amount of hypernegatively topoisomers are detected for both plasmids. This can best be explained by the fact that transcription of the *rrnB Hind*III fragment in the physiological orientation produce R-loops that are more stable than the ones generated in the reverse orientation (8). This stability can be manifested either *in vivo* or during the extraction of the plasmid DNAs and the following treatment with RNase A before electrophoresis (see "Discussion").

One prediction that can be made if the formation of hypernegatively supercoiled plasmid DNAs in *topA* mutants is directly linked to R-loop formation, is that overproducing RNase H should reduce the formation of such topoisomers. To test this hypothesis, we introduced the plasmid pEM001 to overproduce RNase H into the cold-sensitive *topA* mutant carrying pMD306. As a control, pEM003, carrying a mutated *rnhA* gene, was used instead of pEM001. The cells were grown with or without IPTG, as described for the experiment shown in Fig. 5 and the plasmid DNAs were extracted by the alkaline lysis protocol (13). The presence of hypernegatively supercoiled plasmid DNAs was detected by two-dimensional agarose gel electrophoresis in the presence of chloroquine. As can be seen in Fig. 6, a decrease in the amount of hypernegatively supercoiled pMD306 DNA (indicated by an *arrow*) is observed when RNase H is overproduced (compare + IPTG, pEM001 with + IPTG, pEM003). Indeed, densitometry analysis of the two-dimensional gel shown in Fig. 6, indicates that the proportion of such topoisomers decrease by a factor of about 3 when RNase H is overproduced (data not shown). These results suggest that the generation of an important proportion of hypernegatively supercoiled pMD306 DNA in the cold-sensitive *topA* mutant, is
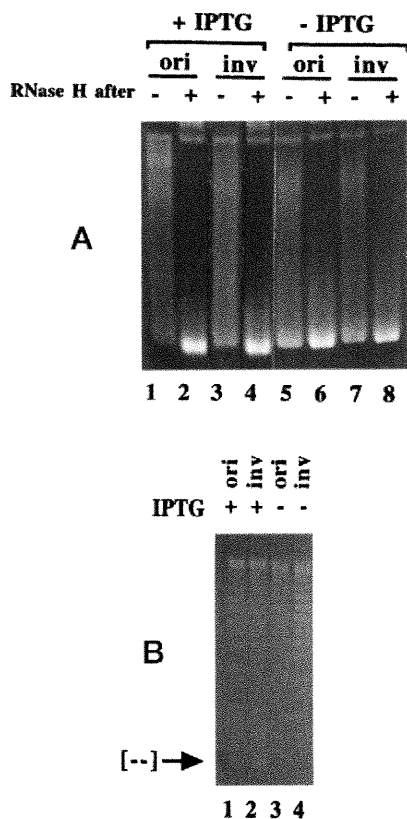
FIG. 5. **Transcription of the 567-bp *rrnB HindIII* fragment in both orientations allows the extraction of R-looped plasmid DNAs and hypernegatively supercoiled plasmid DNAs from RFM480 cells grown at 30 °C.** The cells were first grown at 35 °C to an OD of 0.4 at which time they were transferred to 30 °C and incubated for 75 min in the presence or absence of 1 mM IPTG as indicated. Plasmid DNAs, either pMD306 or pMD308, were extracted as described under "Experimental Procedures." *A*, DNA samples were loaded on an agarose gel (with 0.5 μg/ml chloroquine) to detect R-looped plasmid DNAs as described under "Experimental Procedures." *RNase H after* indicates that RNase H was added (+) or not (−) after the extraction of the plasmid DNAs and therefore, before electrophoresis. *B*, DNA samples were treated with RNase A (1 μg) and RNase H (20 ng) before being loaded on an agarose gel containing 7.5 μg/ml chloroquine. [- -] indicates hypernegatively supercoiled plasmid DNAs. *ori* and *inv*, indicate, respectively, the physiological orientation (pMD306) and the reverse orientation (pMD308) of the 567-bp *rrnB HindIII* fragment relative to the P*trc* promoter.

FIG. 6. **Effect of RNase H overproduction in the *topA*::Tn*10* RFM480 strain on the formation of hypernegatively supercoiled pMD306 DNAs.** The cells were first grown at 35 °C to an OD of 0.4 at which time they were transferred to 30 °C and incubated for 75 min in the presence or absence of 1 mM IPTG as indicated. pMD306 DNA was extracted by the alkaline lysis procedure (13). The DNA samples were analyzed by two-dimensional agarose gel electrophoresis as described under "Experimental Procedures." As presented in this figure, the first dimension (with 7.5 μg/ml of chloroquine) is from the top to the bottom and the second dimension (with 30 μg/ml of chloroquine) is from the left to the right. Under the electrophoresis conditions used the more relaxed topoisomers migrate in the right portion of the curve, whereas the more negatively supercoiled topoisomers migrate in the left portion of the curve. *pEM001* indicates that the cells also carried this plasmid DNA and therefore, that RNase H was overproduced. *pEM003* indicates that the cells also carried this plasmid and therefore, that RNase H was not overproduced. The *arrows* indicate the position of hypernegatively supercoiled plasmid DNAs.

linked to R-loop formation. Similar results were also obtained when the same experiment was performed with pMD308 instead of pMD306 (data not shown). It is, however, obvious that RNase H overproduction *in vivo* do not have the same impact on the production of hypernegatively supercoiled DNA as it has in *in vitro* reactions (8). This can be explained by the fact that other factors *in vivo*, for example, single-stranded binding protein and histone-like proteins, may protect hypernegatively supercoiled DNA from relaxation.

Transcription in Vitro of the 567-bp rrnB HindIII Fragment Allows the Production of R-looped Plasmid DNA—To further support our *in vivo* results and to investigate in greater detail the process of R-loop formation, we designed an *in vitro* transcription system using phage RNA polymerases. These polymerases are highly promoter-specific and will therefore only transcribe DNA sequences placed downstream of an appropriate promoter. We thus subcloned the 567-bp *HindIII* fragment from *rrnB* in both orientation into the pBluescript II KS vector in such a way that it is flanked by promoters for phage T3 and T7 RNA polymerases. In the pJP459 plasmid the *HindIII* fragment is cloned in the reverse
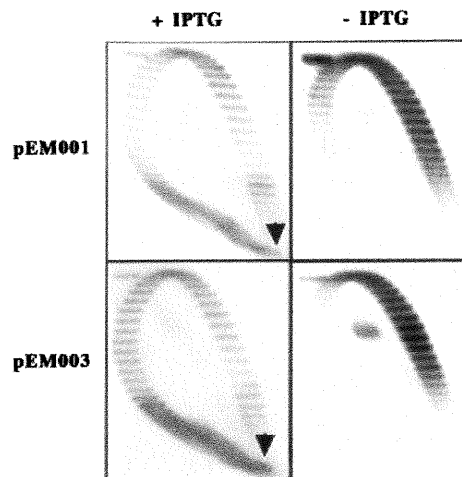
orientation relative to the pBluescript KS map and will thus be transcribed in this orientation by the T7 RNA polymerase and in its physiological orientation by the T3 RNA polymerase. In the pJP461 plasmid the *HindIII* fragment is inverted and will therefore be transcribed in the physiological orientation by the T7 RNA polymerase and in its reverse orientation by the T3 RNA polymerase. As can be seen in Fig. 7, a larger amount of conformers is produced when pJP459 is transcribed by T3 RNA polymerase as compared with transcription by T7 RNA polymerase (compare *lane 1* with *lane 11*), therefore when the *HindIII rrnB* fragment is transcribed in its physiological orientation. This is not an RNA polymerase effect since transcription of pJP461 produces more conformers with T7 RNA polymerase than with T3 RNA polymerase (compare *lane 6* with *lane 16*). Since such conformers are either barely visible or not visible at all after RNase H treatment during or after transcription, they are most likely due to the presence of R-loops (*lanes 3, 4, 8, 9, 13, 14, 18,* and *19*). All these results are therefore in good agreement with the *in vivo* data showing stable R-loop formation when the *rrnB HindIII* fragment is transcribed in its physiological orientation.

Interestingly, the presence of RNase A at 1 μg had almost no effect on R-loop formation, suggesting that the RNA in the R-loop is never free and that it is elongated by RNA polymerase without being displaced from the template strand (compare *lane 1* with *lane 2* and *lane 16* with *lane 17*). A similar conclusion was reached from the results of *in vitro* transcription experiments with pJP459 and pJP461 DNAs, in the presence of DNA gyrase (8). Moreover, increasing the amount of RNase A to 2.5 μg or adding RNase T1 that has a different ribonucleotide specificity, had no effect on R-loop formation (data not shown). In addition, these results with RNase A and T1 to-
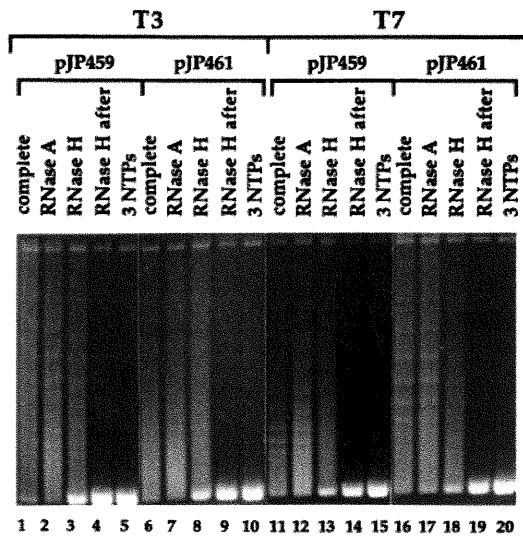
FIG. 7. **R-loop formation during transcription** *in vitro* **in function of the orientation of the 567-bp** *rrnB* **HindIII fragment.** *In vitro* transcription experiments were performed as described under "Experimental Procedures." The DNA samples were all treated with RNase A before electrophoresis as described under "Experimental Procedures." *T3* indicates transcription by phage T3 RNA polymerase and *T7* indicates transcription by phage T7 RNA polymerase. *3 NTPs* means that UTP was omitted in the reaction. *RNase A* and *RNase H* indicate that the corresponding ribonuclease was present during transcription at the concentration indicated under "Experimental Procedures." *RNase H after* means that the samples were treated with RNase H before electrophoresis. In pJP459 the 567-bp *rrnB* HindIII fragment is transcribed in its physiological orientation by T3 RNA polymerase whereas it is transcribed in this orientation by T7 RNA polymerase in pJP461.

gether with the fact that RNase H during transcription strongly decreased the production of conformers (*lanes 3, 8, 13,* and *18*), eliminate the possibility that the production of such structures is an artifact of extraction due to the use of protein denaturants. We also observed that the addition of RNase H during transcription stimulated RNA synthesis, suggesting that an R-loop can act as a road block for RNA polymerases (data not shown).

Since our *in vivo* results suggested that the level of negative supercoiling was an important factor for extensive R-loop formation on the *rrnB* HindIII fragment, we decided to look for R-loop formation during transcription of relaxed pJP459 and pJP461 DNAs. No RNase H-dependent changes in the electrophoretic mobility patterns were observed for any plasmids and polymerases used, suggesting that stable R-loops did not form on relaxed DNAs (data not shown).

DISCUSSION

In the present study we have provided genetic and biochemical evidence that R-loop formation can occur during transcription of the *rrnB* operon in *E. coli.* We first showed that heavy transcription of a portion of the *rrnB* operon was detrimental for the growth of all *topA* mutants tested unless RNase H was overproduced. We then demonstrated the formation of conformers when the 567-bp *rrnB* HindIII fragment was transcribed on a plasmid (pMD306) and in a *topA* mutant, suggesting the presence of R-loops on the plasmid DNAs. Indeed, the disappearance of such conformers upon RNase H treatment confirmed the presence of R-loops. Following these results it was important to show that these R-loops were generated during transcription *in vivo* and therefore, that their formation was not induced during plasmid DNAs extraction. Two sets of experiments strongly suggested that these R-loops were generated during *in vivo* transcription. First, we found that overpro-

ducing RNase H *in vivo* considerably decreased the amount of conformers produced. Second, when DNA gyrase was sufficiently active we were able to detect the presence of hypernegatively supercoiled plasmid DNAs, a predictable event if extensive R-loop formation occurs. This was indeed supported by the finding that the formation of such topoisomers was sensitive to the intracellular level of RNase H. The *in vivo* evidence for R-loop formation during transcription of the *rrnB* HindIII fragment is also supported by the results of *in vitro* transcription experiments: the formation of RNase H-sensitive conformers (this study) and the formation of hypernegatively supercoiled DNA in the presence of DNA gyrase and in a manner sensitive to the level of RNase H activity (8).

A larger amount of conformers, and therefore R-loops, was always detected when the *rrnB* HindIII fragment was transcribed, either *in vivo* or *in vitro*, in its physiological orientation as compared with its reverse orientation. Similar results were recently obtained by measuring the R-loop-dependent linking number change following the addition of DNA gyrase either during or after transcription (8). The results of such experiments allowed us to conclude that roughly the same amount of R-loops were generated in both orientations under the experimental conditions used, but that the R-loops generated in the physiological orientation were more stable than the ones generated in the reverse orientation. This conclusion regarding the stability was based on the sensitivity of the R-loops to RNase A, a ribonuclease specific to single-stranded RNA. By considering this stability factor, the absence of conformers when the *rrnB* HindIII fragment was transcribed in its reverse orientation (pMD308) at 35 °C may be explained by a combination of *in vitro* and *in vivo* factors. It is possible that the unstable R-loops were lost during the extraction of plasmid DNAs and/or that their RNA was degraded by RNase A before loading the samples on the agarose gels. It is predictable and it has indeed been shown that R-loops are generally less stable on relaxed DNA than on negatively supercoiled DNA (1). In this context one can imagine that the absence of conformers on pMD308 extracted from cells grown at 35 °C is due to the fact that the negative supercoiling level was too low (lower than in a wild-type cell; data not shown), whereas the presence of such conformers when the same plasmid was extracted from cells grown at 30 °C is explained by a higher negative supercoiling level which can stabilize the R-loops. Because the R-loops that are produced when the *rrnB* HindIII fragment is transcribed in its physiological orientation tend to be more stable, they will be less affected by the supercoiling level. This would explain why large amounts of conformers were detected on pMD306 extracted from cells grown at both temperatures (30 and 35 °C). By considering the supercoiling factor as a key element in R-loop formation one can explain why a mutation reducing the level of negative supercoiling, such as a mutation in *gyrA* or *gyrB*, will be the best mutation to compensate for the absence of DNA topoisomerase I, since such a mutation will prevent stable R-loop formation. Indeed, R-loop formation was not detected when the *rrnB* HindIII fragment was transcribed in its physiological orientation on a relaxed DNA template *in vitro*. Moreover, by exposing the cold-sensitive *topA* mutant to a temperature higher than 37.5 °C, no R-loops were detected on pMD306 DNA which was also found to be significantly more relaxed than DNA extracted from wild-type cells (data not shown). Therefore, as concluded from the results of *in vitro* experiments (8), our *in vivo* results allow us to suggest that DNA gyrase participates both in R-loop initiation and R-loop elongation by providing the negative superhelical density needed for these processes.

Our results also suggest that the nucleotide sequence of the

DNA template can contribute to the stability of the R-loop. What sequence element is responsible for the higher stability of the R-loop generated during transcription of the *Hind*III *rrnB* fragment in its physiological orientation, is unknown for the moment. Interestingly, it has been shown that a RNA-DNA hybrid with a purine RNA is more stable than a hybrid with a pyrimidine RNA (25). In addition, recent results of *in vitro* experiments have shown that long polypurine stretches can favor the formation of very stable RNA-DNA hybrids if transcription occurs in the direction that makes purine-rich RNA (26, 27). We note that the RNA made when the *rrnB Hind*III fragment is transcribed in its physiological orientation is more purine-rich than the RNA made when it is transcribed in its reverse orientation. Whether it is significant or not remains to be determined. It is also important to mention that transcription of other DNA fragments from the *rrnB* operon and from other sources was recently found to have a detrimental effect on the growth of *topA* mutants in a manner that depends on the intracellular level of RNase H.[4] We therefore believe that the major factor influencing R-loop formation in general is the supercoiling level of the DNA template but that the nucleotide sequence of the DNA template is also an important factor.

Interestingly, in all other *in vitro* systems where R-loop formation has been demonstrated, the primary role (probably the only one) of the transcribed RNA is to form an hybrid with the template DNA strand. Obviously, this is not the case in the present study since the role of the *rrnB* operon is to produce rRNAs and tRNAs. In the other described systems RNA-DNA hybrids are formed either to create RNA primers to initiate DNA replication (2, 28), or possibly to be used as a recombination intermediate in the process of immunoglobulin class switching (26, 29). Therefore, a major question is whether or not R-loop formation at *rrnB* is biologically significant. To be biologically meaningful, R-loop formation during transcription of the *rrnB* operon must occur in wild-type cells. We have found that pNO1302 DNA is not easily maintained in wild-type cells unless RNase H is overproduced. In addition, pNO1302 but not the pEM501 derivative without *rrnB* promoters, was found to contain R-loops when extracted from wild-type cells.[4] These results suggest that even in the presence of negative regulators such as DNA topoisomerase I and RNase H, R-loop formation can occur when *rrnB* transcription increases considerably. This is an indication that the system regulating R-loop formation can be saturated in wild-type cells and therefore, that such structures may form in these cells. Moreover, to be biologically relevant, R-loop formation does not have to be frequent especially when one considers the possible involvement of such structures in initiation of DNA replication and DNA recombination. Interestingly, in the case of the cold-sensitive *topA*

mutants, we repeatedly observe a 10–20-fold increase in the gene duplication rate between different *rrn* operons as compared with the rate in an isogenic *gyrB*(Ts) strain.[5] Whether R-loop formation during transcription of *rrn* operons is directly or indirectly involved in gene duplication remains to be tested. It is worth mentioning that the absence of either type I DNA topoisomerase (Top1 or Top3) in the yeast *Saccharomyces cerevisiae* results in increased recombination in the rDNA multiple tandem array (30–32). The possible involvement of R-loop formation in this process has not yet been investigated.

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7.2 « *Relaxation of transcription-induced negative supercoiling is an essential function of* Escherichia coli *DNA topoisomerase I* » *par Massé et Drolet, (1999a).*

Il a été suggéré que le rôle essentiel de la topoisomérase I chez *Escherichia coli* est d'empêcher que le surenroulement global du chromosome n'atteigne une densité négative trop élevée. Cependant, d'autres études *in vivo* ont démontré que la topoisomérase I était très efficace pour éliminer le surenroulement local généré durant la transcription. Afin de déterminer si le rôle essentiel de la topoisomérase I est de contrôler le surenroulement global ou local, nous avons construit une série de mutants *topA* et utilisé différents plasmides. Nous démontrons qu'il existe une corrélation entre la croissance bactérienne maladive et le surenroulement de l'ADN généré par la transcription de même qu'avec le surenroulement global. L'inhibition presque complète de la croissance ne correspond cependant qu'avec le surenroulement local généré durant la transcription. Ce résultat suggère fortement que le rôle majeur de la topoisomérase I est d'éliminer le surenroulement négatif local produit durant l'élongation de la transcription.

Relaxation of Transcription-induced Negative Supercoiling Is an Essential Function of *Escherichia coli* DNA Topoisomerase I*

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It has been suggested that the essential function of DNA topoisomerase I in *Escherichia coli* is to prevent chromosomal DNA from reaching an unacceptably high level of global negative supercoiling. However, other *in vivo* studies have shown that DNA topoisomerase I is very effective in removing local negative supercoiling generated during transcription elongation. To determine whether topoisomerase I is essential for controlling global or local DNA supercoiling, we have prepared a set of *topA* null mutant strains in combination with different plasmid DNAs. Although we found a correlation between the severity of the growth defect with both transcription-induced and global supercoiling, near to complete growth inhibition correlated only with transcription-induced supercoiling. This result strongly suggests that the major function of DNA topoisomerase I is to relax local negative supercoiling generated during transcription elongation.

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 (1). 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 is to prevent chromosomal DNA from reaching an inappropriate level of negative supercoiling. This model stems from the observation that *topA* null mutants are viable 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 were decreased below the normal level (2, 3). 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, which specifically relaxes negative supercoiling, and DNA gyrase, with two different subunits encoded by *gyrA* and *gyrB* that introduces negative supercoiling. However, exactly how a high level of global negative supercoiling could be detrimental to cell growth is not known.

In vivo and *in vitro* studies have shown that DNA topoisomerase I is highly efficient in removing negative supercoils produced in the wake of moving transcription complexes (4–6). Such supercoiling can be generated during transcription elongation because of the difficulty for a moving transcription complex to rotate around the double helix (the twin-domain model for transcription; see Ref. 7). 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. In some cases, especially when the transcribed genes encode membrane bound proteins, extreme negative supercoiling is generated by transcription (8, 9). When such genes are present on a plasmid DNA, transcription in the absence of DNA topoisomerase I has been shown to generate hypernegatively supercoiled DNA (5, 8–10). 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, one may conclude that the removal of transcription-induced negative supercoiling by DNA topoisomerase I is not essential for cell growth.

In the work reported here, we present data suggesting that the essential function of DNA topoisomerase I is linked to transcription elongation and not to the control of the global level of negative supercoiling. These results were obtained by measuring the linking number deficit of pBR322 derivatives extracted from various *topA* mutants having different growth capacities. On these pBR322 derivatives, the effect of transcription of the *tetA* gene that encodes a membrane bound protein is either kept at its minimum or totally abolished. These plasmid DNAs allowed us to measure both global negative supercoiling and transcription-induced negative supercoiling and to make a correlation between these parameters and the growth of the various *topA* mutants. Our results indicate that the essential function of DNA topoisomerase I is linked to transcription-induced negative supercoiling. Our results presented in Ref. 11 further suggest that one detrimental consequence of the failure to relax transcription-induced supercoiling is R-loop formation (11).

EXPERIMENTAL PROCEDURES

E. coli Strains—The *E. coli* strains used are listed and described in Table I. DM800 derivatives were constructed by P1*vir* transduction (12). The RFM475 cold-sensitive strain is well described by Drolet *et al.* (13).

Plasmids—pBR322ΔPtet is a pBR322 derivative with a small deletion within the *tetA* promoter region (5). This deletion was further characterized in the present study by sequencing the appropriate region of the plasmid (Fig. 1). pBR322Δtet5' was obtained by deleting the *Hind*III-*Eco*RV DNA fragment from pBR322. The *Hind*III-*Eco*RV-digested pBR322 vector was treated with the Klenow enzyme to fill in the *Hind*III site, before being treated with DNA ligase. Transformants were selected on LB medium with ampicillin and were screened for sensitivity to tetracycline. The plasmid DNA of some Tet^s transformants was

Strains	Genotype	Reference/construction
RFM475	*rpsL galK2, Δlac74, gyrB221*(cou^R), *gyrB203*(Ts) Δ(*topA cysB*)204	13
DM800	Δ(*topA cysB*)204 *gyrB225, acrA13*	2, 3
CAG18592	*zie-3163*::Tn*10kan*	14
RFM445	*rpsL galK2, Δlac74, gyrB221*(cou^R), *gyrB203*(Ts)	13
PH326	RFM445 *zie-3163*::Tn*10kan*	RFM445XP1.CAG18592, select Km^r, Cou^r and Ts
EM169	DM800 *gyrB221*(cou^R), *gyrB203*(Ts)	DM800XP1.PH326, select Km^r, Cou^r and Ts
EM176[a]	DM800 *gyrB$^+$*	DM800XP1.CAG18592, select Km^r, small colonies and white colonies in the presence of 5-bromo-4-chloro-3-indolyl β-D-glucoside

[a] The *gyrB$^+$* selection is based on the observation that *gyrB$^+$* cells are Bgl$^-$, whereas *gyrB225* cells are Bgl$^+$ due to the activation of the cryptic *bgl* operon (3). Bgl$^+$ cells will be able to use the β-glucoside analog 5-bromo-4-chloro-3-indolyl β-D-glucoside and form blue colonies, whereas Bgl$^-$ cells will not use this analog and will form white colonies.

analyzed by using appropriate restriction enzymes to confirm the *Hin*dIII-*Eco*RV deletion.

Media and Growth Conditions—Unless otherwise indicated, bacteria were grown in VB Casa or LB medium (13) supplemented with cysteine (50 μg/ml) at the temperature indicated in the table and figure legends. When needed, antibiotics were added as follows: ampicillin at 50 μg/ml, and chloramphenicol at 30 μg/ml. Because of the *acrA13* mutation in the DM800 derivatives, which renders these cells more permeable to many antibiotics (3), chloramphenicol was used at 10 μg/ml for these cells. Isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl β-D-glucoside were purchased from Sigma.

For the extraction of plasmid DNAs for supercoiling analysis, bacterial cells carrying various pBR322 derivatives were grown overnight in VB Casa medium at 37 °C and then diluted 1/75 in prewarmed LB medium. The cells were grown to an A_{600} of 0.4 at 37 °C at which time they were transferred to the desired temperature. The plasmid DNAs were extracted when the A_{600} reached about 0.7 or after an exposition of about 2 h at the respective temperature when an A_{600} of 0.7 could not be reached. We found that 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. Growth was stopped by transferring the cells in a tube filled with ice. By this procedure, the temperature of the cultures immediately dropped to 0 °C. Plasmid DNAs were extracted by an alkaline lysis procedure (15).

Current Molecular Biology Techniques—The current molecular biology techniques were performed essentially as described (16). CaCl$_2$ transformations were carried out as described by Drolet *et al.* (13).

Electrophoresis in the Presence of Chloroquine—One-dimensional and two-dimensional agarose gel electrophoresis in the presence of chloroquine in 0.5 × TBE were performed essentially as described (16). Chloroquine was used at the concentration indicated in the figure legends. After electrophoresis, agarose gels were dried and prepared for *in situ* hybridization as described (16).

RESULTS

A Genetic System to Correlate Local and Global Negative Supercoiling Fluctuations with Cell Growth in the Absence of DNA Topoisomerase I—Most of the previous studies related to the effects of transcription on DNA supercoiling were designed to support the twin-domain model for transcription (7) but not to test if DNA topoisomerase I activity during transcription is crucial for cell growth. In these studies, transcription of genes encoding membrane bound proteins allowed the extraction of hypernegatively supercoiled plasmid DNAs from *topA* null mutants (5, 8, 9). Although the results of these studies supported very well the twin-domain model for transcription, the question of DNA topoisomerase I activity being essential during transcription was not addressed or could not even be addressed, because the *topA* null mutants used in these studies grew very well. Indeed, the fact that these strains grew very well may suggest that DNA topoisomerase I activity during transcription is not normally essential.

Therefore, to better address the role of DNA topoisomerase I in transcription elongation, we had to design a genetic system in which at least two requirements needed to be met: 1) the plasmid DNAs used should not carry genes encoding for mem-

brane bound products; 2) the *topA* mutants used should not benefit from compensatory mutations in order for the *topA* phenotypes to be fully expressed. Because most of the results supporting the twin-domain model for transcription were obtained by using pBR322 DNA, we decided to use several of its derivatives in our studies. In the early studies, the extraction of hypernegatively supercoiled pBR322 DNA from *topA* null mutants carrying compensatory mutations, was clearly shown to be dependent on *tetA* gene expression, which encodes a membrane-bound protein (9). One derivative used in one study, pBR322ΔP*tet*, has a deletion within the promoter responsible for *tetA* expression (Fig. 1A), which was shown to abolish the production of hypernegatively supercoiled pBR322 DNA (5). We therefore decided to use this pBR322 derivative in our studies. However, as can be seen in Table II, this plasmid confers residual tetracycline resistance, because bacterial cells carrying it can grow in the presence of 4 μg/ml tetracycline, whereas cells carrying no plasmid DNAs can only grow when tetracycline concentrations do not exceed 1 μg/ml. This residual *tetA* expression can be explained by the fact that a weak −10 promoter region, according to the consensus, was created during plasmid construction (Fig. 1B). We constructed an additional pBR322 derivative, pBR322Δ*tet5'* (Fig. 1A), from which *tetA* gene expression is not detectable (Table II). This plasmid has an *Hin*dIII-*Eco*RV deletion, removing the 5' part of the gene including the original −10 promoter region, the Shine-Dalgarno sequence, the ATG initiator codon, and one transmembrane domain responsible for the anchorage of the TetA protein to the membrane (Fig. 1A). Interestingly, by performing such a deletion, a −10 region that restored a promoter sequence was produced (Fig. 1B). Indeed, promoter activity was detected when the *lacZ* gene was cloned downstream in the appropriate orientation (Fig. 1B).

The relative growth capacity of the various *topA* null strains used in our studies is shown in Table III. It can be seen that the strain showing the most severe growth defect and therefore, in our view, the strain that most closely resembles a true *topA* null mutant without compensatory mutations is RFM475. Low temperatures are more restrictive for this strain because of the *gyrB*(Ts) allele that regains a more wild-type level of activity at these temperatures (13). The other strain that grows poorly is a *gyrB*+ derivative of the widely used DM800 strain (Δ*topA* and *gyrB225*). The fact that it is possible to introduce a wild-type *gyrB* allele in DM800 indicates a possible presence of additional compensatory mutation(s) as previously considered when a similar transduction experiment was performed (3). Moreover a *gyrB*(Ts) derivative of DM800, a strain that should almost be identical to RFM475, grows significantly better than RFM475 (Table III), again suggesting that additional compensatory mutation(s) might be present. It is also important to note that all the Δ*topA* strains used in our studies carry the same *topA* deletion [Δ(*topA cysB*)204].
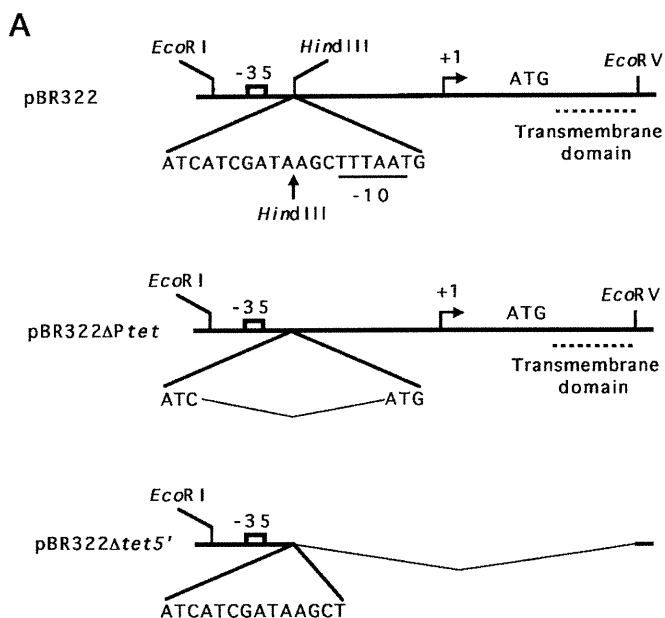
A

B

```
TTGACAgcttatcatcgataagcTTTAAT     pBR322

TTGACAgcttatcatgcggtagttTATCAC    pBR322ΔPtet

TTGACAgcttatcatcgataagcTATCGT     pBR322Δtet5'

TTGACA-----16 to 19----TATAAT     Consensus
-35                     -10
```

FIG. 1. **The *tetA* promoter region of the various pBR322 derivatives used in this study.** In *panel A*, a schematic drawing of the 5' *tetA* region of the various pBR322 derivatives used in this study is shown. −35 and −10 represent the respective consensus sequences for σ70 *E. coli* promoters (17), +1 shows the transcriptional start site of *tetA*, and ATG is the initiator codon of the TetA protein. One transmembrane domain responsible for the anchorage of the TetA protein to the membrane and hence responsible for generating hypernegatively supercoiled pBR322 DNA in *topA* mutants (9) is also indicated. The deleted region within the various pBR322 derivatives is indicated by *thin lines*. In *panel B*, the nucleotide sequence of the *tetA* promoter region of the various pBR322 derivatives used in this study is shown. For both pBR322ΔPtet and pBR322Δtet5' the promoter sequence that was reconstituted due to the deletion is shown. The presence of promoter activity for pBR322Δtet5' was detected when the *lacZ* gene was cloned in the appropriate orientation within the *Bam*HI cloning site of this plasmid (data not shown). Letters in *bold type* reflect the most frequently found nucleotides at these positions (17). 16 to 19 indicates the number of nucleotides separating the −35 and −10 regions, with 17 being the most frequently found number.

TABLE II
Growth of RFM475 carrying pBR322 derivatives on LB plates with various amounts of tetracycline

RFM475 cells carrying the indicated plasmid were grown overnight in liquid VB Casa medium with cysteine and ampicillin (except for the strain carrying no plasmid) at 37 °C, and 2 μl were streaked on LB plates with the indicated concentration of tetracycline. The plates were incubated for 20 hrs at 37 °C. +, reflects colony size; −, absence of growth.

Plasmids	Tetracycline				
	0	1	2	4	6
			μg/ml		
No plasmid	+ +	+	−	−	−
pBR322	+ +	+ +	+ +	+ +	+ +
pBR322ΔPtet	+ +	+ +	+ +	+	−
pBR322Δtet5'	+ +	+	−	−	−

The Formation of Transcription-induced Hypernegatively Supercoiled Plasmid DNA Correlates with the Severity of the Growth Defect in the Absence of DNA Topoisomerase I—The

TABLE III
Relative colony size on LB medium of the various ΔtopA strains used in this study

Bacterial cells were grown overnight in liquid VB Casa medium with cysteine at 37 °C, and 2 μl were streaked on LB plates that were incubated at 37, 28, and 21 °C, respectively, for 20, 42, and 72 h (EM176 and RFM475 were incubated for 6 days at 21 °C). +, reflects colony size; −, absence of growth.

Bacterial strains	Temperature		
	37 °C	28 °C	21 °C
DM800 [ΔtopA, gyrB225]	+ + + +	+ + + +	+ + + +
EM176 [DM800 gyrB+]	+	+	1/2
EM169 [DM800 gyrB(Ts)]	+ + + +	+ + 1/2	+ +
RFM475 [ΔtopA, gyrB(Ts)]	+ +	−*a*	−

a The few colonies that appeared are made of bacterial cells with *tolC* region duplications known to compensate for the absence of *topA*, as previously described for this bacterial strain (13).

various ΔtopA mutants carrying pBR322ΔPtet were grown in LB medium at 37 °C and exposed to the indicated temperatures as described under "Experimental Procedures." All the strains were exposed to various temperatures to discriminate between true temperature effects and allele specific effects [gyrB(Ts)] on DNA supercoiling. The extracted plasmid DNAs were subjected to electrophoresis in agarose gel in the presence of chloroquine at 7.5 μg/ml, as described under "Experimental Procedures." Under these conditions the more negatively supercoiled topoisomers migrate slowly except for the fastest migrating band pointed to by an arrow (Fig. 2A, *lane 12* [- -]), which represents hypernegatively supercoiled plasmid DNAs. It can be seen that the global DNA supercoiling level in the various strains, represented by the topoisomers distributions of pBR322ΔPtet DNA without considering hypernegatively supercoiled DNA, reflects very well the level of gyrase activity within these strains (Fig. 2A). Indeed, plasmid DNAs extracted from the DM800 strain that carries the gyrB225 mutation are less negatively supercoiled than plasmid DNAs extracted from the DM800 gyrB+ derivative (EM176) or the gyrB(Ts) strains (EM169 and RFM475) exposed to temperatures of 28 °C and below (Fig. 2A). It is also obvious that the global negative supercoiling level correlates with the growth defects (Table III). However, this correlation is not complete because the global negative supercoiling level eventually reaches a maximum value even though some bacterial strains are still growing, albeit slowly (EM169 at 28 °C and 21 °C, EM176 at 28 °C, and very slowly at 21 °C), whereas others are not (RFM475 at 28 °C (almost undetectable growth) and 21 °C). Under more restrictive conditions, when the ΔtopA mutant completely fails to grow (RFM475 at 21 °C), hypernegatively supercoiled pBR322ΔPtet DNA is found (Fig. 2, A, *lane 12*; B, *bottom right panel*). The formation of such topoisomers is completely dependent on transcription, because it is abolished by rifampicin treatment (data not shown). Such topoisomers are not found when pBR322ΔPtet DNA is either extracted from DM800 carrying the gyrB+ allele (Fig. 2, A, *lanes 4-6*; B, *top middle panel*), or DM800 carrying the gyrB(Ts) allele and grown at 21 °C (Fig. 2, A, *lane 9*; B, *top right panel*), again supporting our conclusion about the presence of additional compensatory mutation(s) within DM800. Similar results are also obtained when the same set of experiments are performed with pBR322Δtet5' (Fig. 3). A larger proportion of hypernegatively supercoiled topoisomers was detected when pBR322Δtet5' DNA was extracted from RFM480 grown at 21 °C (and a small amount from cells grown at 28 °C), a strain identical to RFM475, but carrying a topA::Tn10 allele instead of the [Δ(topA cysB)204] found in RFM475 (11). This is possibly due to the fact that RFM480 is genetically more stable than RFM475.
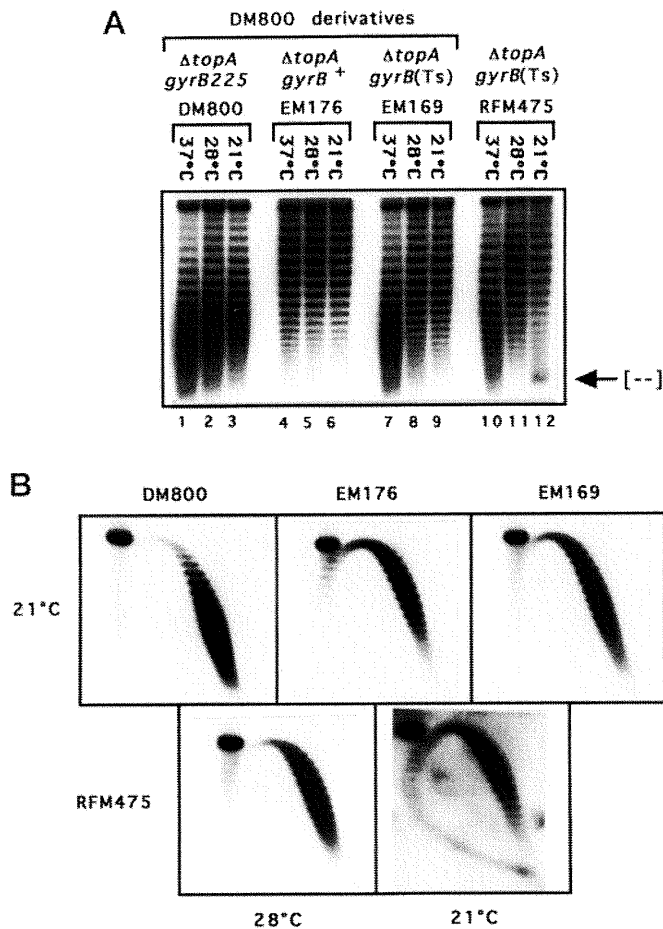
FIG. 2. **Transcription-induced hypernegative supercoiling of pBR322ΔP*tet* DNA in various *topA* null mutant.** The various *topA* null mutants carrying pBR322ΔP*tet* were grown, and the plasmid DNAs were extracted as described under "Experimental Procedures." In *panel A*, the samples were analyzed by electrophoresis in an agarose gel containing 7.5 μg/ml of chloroquine. [- -] indicates hypernegatively supercoiled topoisomers. In *panel B*, some samples were also 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.

DISCUSSION

Our results show that severe growth inhibition of *Escherchia coli* in the absence of DNA topoisomerase I correlates with transcription-induced negative supercoiling, but not with global negative supercoiling. Therefore, it suggests that *topA* mutants fail to grow when DNA gyrase is too active during the process of transcription elongation. Under these conditions, DNA gyrase activity is efficient whether or not genes encode for membrane bound proteins. In fact, under these conditions, we have also detected hypernegatively supercoiled topoisomers for several others plasmid DNAs that do not carry genes encoding for membrane bound proteins. For the moment, we cannot exclude the possibility that the apparent increase in DNA gyrase activity at low temperatures is not, for unknown reasons, only linked to the *gyrB*(Ts) allele, but to the low temperature itself. Indeed, our preliminary data with DM800 derivatives suggest that this might be the case.[1] Either way, the result of this activity, if not counteracted by DNA topoisomerase I, is inhibitory to cell growth. The results presented in the accompanying manuscript (11) show that extensive R-loop for-
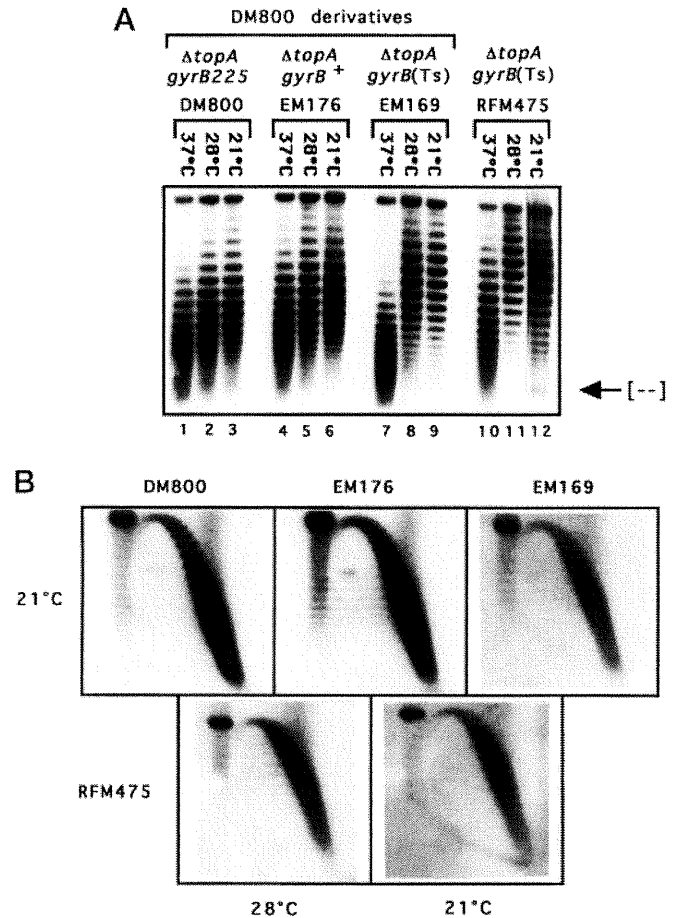

FIG. 3. **Transcription-induced hypernegative supercoiling of pBR322Δ*tet5′* DNA in various *topA* null mutant.** The various *topA* null mutants carrying pBR322Δ*tet5′* were grown, and the plasmid DNAs were extracted and analyzed as described in the legend to Fig. 2.

mation can occur under these conditions (11). This is most likely inhibitory to cell growth, because the cellular level of RNase H activity must be properly increased to support growth under conditions where *topA* mutations are fully expressed (13).

Our results also strongly suggest that *gyr* mutations arose in *topA* mutants to reduce gyrase activity during transcription elongation. We can also conclude that the increase in the level of global negative supercoiling in some *topA* mutants (see Ref. 1 and this work), is only a secondary consequence of the absence of DNA topoisomerase I and is not linked to the essential function of this enzyme. This notion is further supported by our recent observations that the overproduction of both RNase H (11) and topoisomerase III (TopB)[2] can very well correct the growth defect of *topA* null mutants without, however, altering the global supercoiling level.

In conclusion, although the previous studies with various *topA* null mutants have been very useful to reveal the regulatory potential of negative supercoiling on DNA functions, they did not reveal the essential function of DNA topoisomerase I. In our view, this enzyme should be considered, at least in part, as a transcription factor and not as a regulator of the global supercoiling level.

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[1] E. Massé and M. Drolet, unpublished results.

[2] S. Broccoli, P. Phoenix, E. Massé, and M. Drolet, manuscript in preparation.

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7.3 « **Escherichia coli** *DNA topoisomerase I inhibits R-loop formation by relaxing transcription-induced negative supercoiling* » *par Massé et Drolet, (1999b).*

Il a été démontré récemment que la surproduction de la RNase H pouvait partiellement corriger le défaut de croissance des mutants *topA* chez *Escherichia coli* (Drolet *et al.*, 1995). Ce résultat a permis d'élaborer un modèle suggérant la formation de R-loops inhibiteurs durant la transcription chez les mutants *topA*. Les résultats présentés dans ce travail supportent ces affirmations et démontrent que le surenroulement de l'ADN généré durant la transcription est impliqué dans le processus de formation des R-loops. Premièrement, nous démontrons que la formation de R-loops stables durant la transcription *in vitro* par la polymérase ARN de *E. coli* ne se produit qu'en présence de la gyrase. Deuxièmement, la formation de R-loops ne s'observe que lorsque les mutants *topA* ne peuvent croître. Finalement, nous démontrons que le couplage de la transcription avec la traduction est un moyen efficace de prévenir la formation de R-loops.

Escherichia coli DNA Topoisomerase I Inhibits R-loop Formation by Relaxing Transcription-induced Negative Supercoiling*

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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 nontemplate 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, pBR322ΔP*tet* has a small deletion within the promoter of the *tetA* gene that considerably reduces its expression. In pBR322Δ*tet5'* an *Hind*III-*Eco*RV 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 *rnhA* 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 MgCl$_2$, 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 HI 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 M 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 MgCl$_2$, and 0.1 M NaCl 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 MgCl$_2$, 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*::Tn*10*, *gyrB221*(cou^R), *gyrB203*(Ts), Ref. 5) carrying the various pBR322 derivatives were grown overnight in VB Casa 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 A_{600} of 0.4 at 37 °C at which time they were transferred to the desired temperature. The plasmid DNAs were extracted when the A_{600} reached about 0.7 (all the strains at 37 °C and only the ones carrying pEM001 at 28 °C and therefore overproducing RNase H) or after an exposition of 2 h at the respective temperature when an A_{600} of 0.7 could not be reached (the strains that do not overproduce 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 × 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 *topA* 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 vivo* (8). To detect R-loop formation, we used the previously described assay in which RNase H-sensitive gel retardation

FIG. 1. **R-loop formation 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 were added during transcription. Samples in *lanes 5* and *8* were treated with RNase H after transcription. Samples were analyzed by electrophoresis in agarose gels in the presence (*panel B*) or absence (*panel A*) of chloroquine at 1 µg/ml.

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-

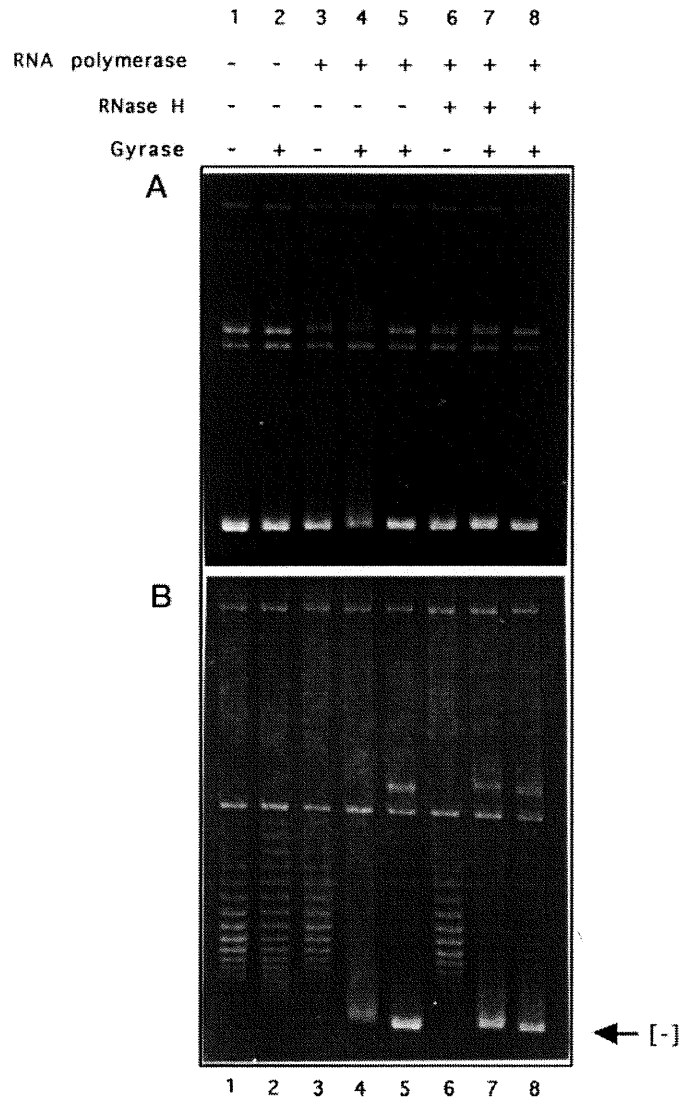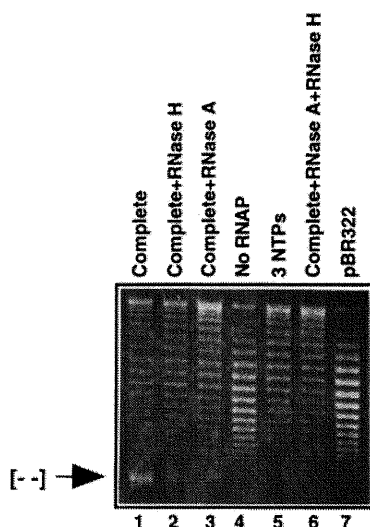FIG. 2. **Hypernegative supercoiling during *in vitro* transcription with *E. coli* RNA polymerase.** The transcription reactions were performed as described under "Experimental Procedures." When indicated, 7.5 ng of RNase H and/or 1 μg of RNase A were added during transcription. 3 NTPs means that GTP 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.

sents 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 template 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).[1]

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*::Tn*10* 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 regains 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, pBR322ΔP*tet* (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 pBR322ΔP*tet* confers low level tetracycline resistance, because a weak promoter was reconstituted during the construction of this plasmid. When this plasmid was ex-

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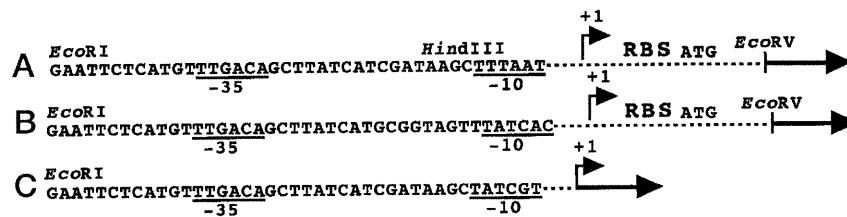*Escherichia coli RNA Polymerase in R-loop Formation*

FIG. 3. **The 5' *tetA* gene region of the various pBR322 derivatives used in this study.** The 5' *tetA* region of *A* (pBR322), *B* (pBR322ΔP*tet*) and *C* (pBR322Δ*tet5'*). −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 pBR322Δ*tet5'* was confirmed when the *lacZ* gene was cloned downstream in the appropriate orientation.

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 pBR322ΔP*tet* 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 hypernegative supercoiling of pBR322ΔP*tet* 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 pBR322Δ*tet5'*, 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 initiator 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 pBR322Δ*tet5'* 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 inactivated *rnhA* gene, within RFM480 bearing pBR322Δ*tet5'*. 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 to electrophoresis 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 supercoiled plasmid DNAs. First of all, it can
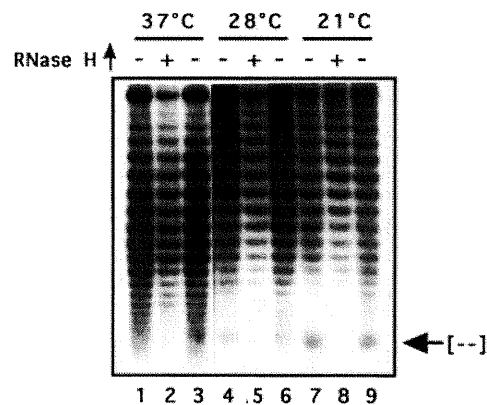

FIG. 4. **The formation of RNase H-sensitive hypernegatively supercoiled plasmid DNAs in a cold-sensitive *topA* null mutant, one-dimensional gel analysis.** RFM480 cells carrying pBR322Δ*tet5'* were grown, and the plasmid DNAs were extracted as described under "Experimental Procedures." In lanes *1*, *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 ^{32}P-labeled DNA fragment carrying the *bla* gene of pBR322.

be seen that the global DNA supercoiling level in the various strains, represented by the topoisomers distributions of pBR322ΔP*tet* 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 pBR322ΔP*tet* DNA, RNase H overproduction abolished the generation of hypernegatively supercoiled pBR322Δ*tet5'* 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 pBR322Δ*tet5'* DNA. Two-dimensional agarose gel analysis was also performed to confirm the presence of hypernegatively supercoiled pBR322Δ*tet5'* 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 pACYC184Δ*tet5'* (*bottom panels*), a pACYC184 derivative carrying an identical deletion to the one found in pBR322Δ*tet5'*. The *tetA* gene is the only common DNA se-
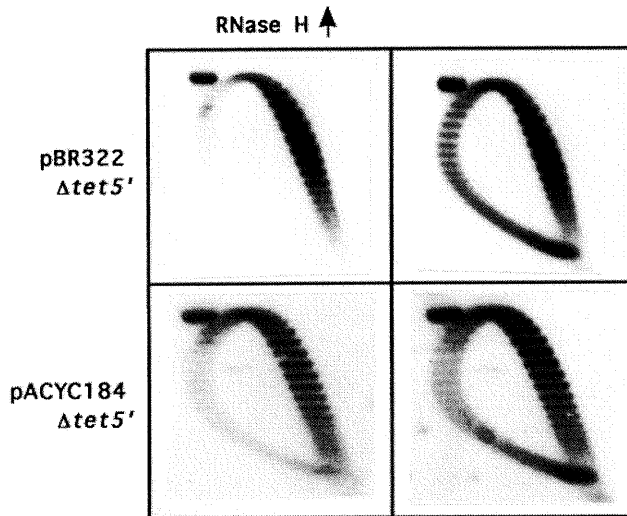
FIG. 5. **The formation of RNase H-sensitive hypernegatively supercoiled plasmid DNAs in a cold-sensitive *topA* null mutant, two-dimensional gel analysis.** RFM480 cells carrying either pBR322Δ*tet5'* (*top panels*) or pACYC184Δ*tet5'* (*bottom panels*) were grown, and the plasmid DNAs were extracted 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 pACYC184Δ*tet5'* (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 pBR322Δ*tet5'* was analyzed, the gel was probed with a ^{32}P-labeled DNA fragment carrying the *bla* gene of pBR322. When pACYC184Δ*tet5'* was analyzed, the gel was probed with a ^{32}P-labeled DNA fragment carrying the *cat* gene of pACYC184.

quence between pACYC184 and pBR322. As it is the case for pBR322 when the *tetA* gene of pACYC184 is translated, the formation of hypernegatively supercoiled plasmid DNA is not sensitive to RNase H overproduction (data not shown).

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 *Eco*RI-*Eco*RV 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
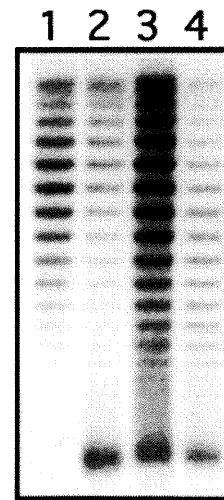

FIG. 6. **Sensitivity of hypernegative supercoiling of pBR322 DNA to the protein synthesis inhibitor, spectinomycin.** RFM480 cells carrying pBR322 were grown to an A_{600} of 0.4 at which time spectinomycin (500 μg/ml) was added (*lanes 1* and *2*) or not (*lanes 3* and *4*). The cells were incubated for an additional 15 min at 37 °C before being exposed to 21 °C. An aliquot of cells was rapidly withdrawn for plasmid DNAs 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 topoisomers 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 topoisomers distribution is more heterogeneous and continuous, as previously shown for pBR322 DNA extracted from various *topA* mutants (11, 13, 15).

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 pBR322ΔP*tet* 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.[1] 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 ColE1 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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7.4 *« R-loop-dependent hypernegative supercoiling in* **Escherichia coli topA** *mutants preferentially occurs at low temperature and correlates with growth inhibition » par Massé et Drolet, (1999c).*

Nous avons récemment proposé que la formation de R-loops inhibiteurs de croissance, survenait lorsque l'ARN naissant s'appariait à l'ADN derrière la polymérase ARN en mouvement. Ceci était supporté par des résultats de transcription *in vitro* et par la démonstration *in vivo* que la formation de R-loops pouvait être prévenue par le couplage de la transcription avec la traduction. Les résultats présenté ici supportent ce modèle et démontrent le lien entre la formation de R-loops et l'inhibition de la croissance des mutants *topA*. Premièrement, nous montrons que l'activité de la RNase H est essentielle en absence de la topoisomérase I. Ceci est observé malgré le fait qu'un mutant *topA* possède d'excellentes mutations compensatoires spontanées qui permettent également une diminution du surenroulement global de l'ADN sous le niveau sauvage. Deuxièmement, nous montrons que la formation de R-loops ainsi que l'inhibition de croissance des mutants *topA* se manifestent d'avantage à basse température. La surproduction de la RNase H à 21°C montre un effet négatif sur la croissance cellulaire. Il est probable que plusieurs ARNm soient emprisonnés dans des R-loops et que leur dégradation par la RNase H affecte significativement la synthèse protéique. Nous proposons que la réduction de la vitesse de transcription due à la baisse de température, favorise l'appariement de l'ARN naissant avec le brin matrice derrière la polymérase. Finalement, nous présentons un modèle qui explique la sensibilité des mutants *topA* aux différentes modifications environnementales qui sont souvent accompagnées d'une inhibition momentanée de la traduction.

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R-loop-dependent Hypernegative Supercoiling in *Escherichia coli topA* Mutants Preferentially Occurs at Low Temperatures and Correlates with Growth Inhibition

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We have recently presented evidence that inhibitory R-loops form during transcription in *topA* null mutants when the nascent RNA anneals with the template DNA strand behind the moving RNA polymerase. This was supported by the results of *in vitro* transcription assays and by *in vivo* studies in which R-loop formation was shown to be inhibited by coupled transcription-translation. The results presented here support this model and further demonstrate the link between R-loop formation and growth inhibition of *topA* null mutants. First, we show that RNase H activity is essential in the absence of DNA topoisomerase I. This was observed even if the growth of the *topA* null mutant is compensated for by naturally selected mutations, that also reduce global supercoiling below the wild-type level. Second, we show that R-loop-dependent hypernegative supercoiling increases as the temperature decreases and correlates with growth inhibition of *topA* null mutants. In fact, RNase H overproduction is shown to be detrimental to cell growth at 21 °C. Presumably, several mRNAs are being sequestered in R-loops and their degradation by RNase H significantly impedes protein synthesis. We propose that a reduced transcription velocity at low temperatures favors the annealing of the nascent RNA with the template strand behind the moving RNA polymerase, in agreement with the results of previous studies. Finally, based on the currently available data on R-loop formation, we present a model that explains the sensitivity of *topA* null mutants to various environmental changes that are often accompanied by transient inhibition of translation.

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Keywords: DNA topoisomerase I; transcription elongation; R-loop; transcription-translation coupling

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Introduction

Escherichia coli DNA topoisomerase I, the first DNA topoisomerase to be discovered (Wang, 1971), was initially shown to specifically relax negatively supercoiled DNA. This substrate specificity was later explained by the fact that this topoisomerase binds to single-stranded DNA regions close to double-stranded ones (Kirkegaard & Wang, 1985). An appropriate level of negative supercoiling within the chromosomal DNA can

promote the formation of such structures, since negative supercoiling favors DNA opening. The process of transcription elongation, in the frame of the twin-domain model, can provide such a high level of negative supercoiling. According to this model, domains of negative and positive supercoiling are transiently generated, respectively, behind and ahead of the moving transcription complex (Liu & Wang, 1987). This model has been supported by much experimental evidence that has also clearly implicated the process of transcription elongation in the generation of hot spots for DNA topoisomerase I activity (Wu *et al.*, 1988; Tsao *et al.*, 1989; Ostrander *et al.*, 1990; Drolet *et al.*, 1994). 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 (Phoenix *et al.*, 1997).

Recent *in vivo* studies have shown that the growth problem of *topA* (encoding for DNA topoisomerase I) null mutants can be partially corrected by overproducing RNase H, an enzyme that degrades the RNA moiety of an R-loop (Drolet *et al.*, 1995). These results have suggested that one major problem in the absence of DNA topoisomerase I is R-loop formation. A correlation was found between the level of DNA gyrase activity, the enzyme responsible for the introduction of negative supercoiling within the chromosomal DNA, 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 (in *gyrA* or *gyrB*) that reduce DNA gyrase activity and correct their growth problem (DiNardo *et al.*, 1982), was therefore explained by the supercoiling activity of DNA gyrase promoting R-loop formation (Drolet *et al.*, 1995). 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 the results of *in vitro* experiments (Drolet *et al.*, 1994; Phoenix *et al.*, 1997; Massé *et al.*, 1997). More recently, a correlation between R-loop formation during transcription and growth inhibition of *topA* null mutants has been shown. This clearly 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 (Massé & Drolet, 1999b). Transcription elongation was strongly suggested to be the key factor underlying the action of both DNA topoisomerases in R-loop formation. Indeed, it was shown that one major role of DNA topoisomerase I is to relax transcription-induced negative supercoiling to inhibit R-loop formation (Massé & Drolet, 1999a,b). In addition, the key action of DNA gyrase in promoting R-loop formation was shown to be at the level of transcription elongation by providing the driving force for R-loop elongation (Massé & Drolet, 1999b). The results of these studies have also suggested that transient local supercoiling is the major problem in the absence of DNA topoisomerase I, as opposed to global supercoiling that reflects the average super-helical density of all supercoiling domains (Massé and Drolet, 1999a). Moreover, this model, in which DNA topoisomerase I acts during transcription or as soon as R-loop formation is initiated, is consistent with the substrate specificity of the enzyme, as described above.

Our recent studies have also revealed the key roles played by the nascent RNA in R-loop for-

mation (Massé & Drolet, 1999b). In addition to being involved in the generation of negative supercoiling during transcription elongation in the frame of the twin-domain model (Liu & Wang, 1987), the nascent RNA is also directly involved in R-loop formation. Indeed, it is the nascent RNA that reanneals with the template DNA strand behind the moving RNA polymerase to initiate R-loop formation. This model implies that the coupling of translation and transcription in bacteria, and hence the binding of ribosomes to the nascent RNA, should inhibit R-loop formation. This was strongly suggested by the fact that R-loop formation on pBR322, as measured by the generation of RNase H-sensitive hypernegatively supercoiled DNA, was shown to occur in the absence of translation in *topA* null mutants (Massé & Drolet, 1999b). In addition, the formation of R-loops upon transcription of a portion of the *rrnB* operon, encoding an untranslated RNA (ribosomal RNA), was observed in the absence of DNA topoisomerase I (Massé *et al.*, 1997). During the course of our studies with *topA* null mutants carrying different compensatory mutations, we noticed that the growth problem of a mutant without any known temperature-sensitive mutations was exacerbated at low temperatures (Massé & Drolet, 1999a). Consistent with this observation, results presented here strongly suggest that R-loop formation is favored at low temperatures. We also show that *topA* null mutants are sensitive to low temperatures in a manner that is linked, at least in part, to R-loop formation. Together with our previous results, the data presented in this report allows us to propose a model that explains the sensitivity of *topA* null mutants to various environmental changes.

Results

RNase H activity is essential in the absence of DNA topoisomerase I irrespective of the strain genetic background and the global supercoiling level

Our previous results suggested that local supercoiling generated during transcription is the key determinant in R-loop formation as opposed to the global level of negative supercoiling. This question was further addressed by performing genetic experiments. If the global level of negative supercoiling is the key determinant in R-loop formation and, therefore, is linked to the mechanism by which DNA topoisomerase I exerts its regulatory function, the reduction of this global level of negative supercoiling below the wild-type level should prevent R-loop formation. In this situation the *rnhA* gene, a normally non-essential gene, should also not be required for the survival of *topA* null mutants. However, the experiments described below show that this is not the case. In fact, we have previously shown that it is not possible to inactivate the *rnhA* gene of strains carrying *topA* null alleles and a *gyrB*(Ts) allele (RFM475, Δ*topA*

and RFM480, *topA20*::Tn*10*), even when the trans- duction experiments were performed at 37 °C, a temperature at which the global level of negative supercoiling within these strains is below the wild- type level (Drolet *et al.*, 1995). Not only has it not been possible to inactivate the *rnhA* gene, but the wild-type level of RNase H is apparently not suffi- cient to promote adequate growth of these strains at 37 °C, since growth is stimulated by RNase H overproduction (Drolet *et al.*, 1995).

Despite the strong evidence showing that the global supercoiling level is not the key determinant in *topA* compensation and hence for R-loop for- mation, we thought that it would still be of interest to test the *rnhA* status within the widely used DM800 strain (Table 1). One reason to perform this experiment is linked to the fact this strain grows very well for a *topA* null mutant, and its compen- satory mutation(s) occurred naturally, which is not the case for RFM475 and RFM480. Moreover, this strain has been extensively studied at the level of cellular DNA supercoiling. These studies have clearly shown that both global chromosomal and plasmid supercoiling levels were similar to each other within this strain and, more importantly, were clearly below the supercoiling level prevail- ing in wild-type cells (Pruss *et al.*, 1982). We thus tried to introduce an *rnhA* null allele within DM800 and its *topA*⁺ derivative, EM205. Transduc- tion experiments were performed with the *rnhA*::- *cam* null allele (*rnh-339*::*cat*) either by directly selecting for chloramphenicol resistants (Cm^R), or by first selecting for the nearby marker *zag- 3198*::Tn*10kan* (kanamycin resistants, Kan^R) and then scoring for the number of Cm^R among the Kan^R. The result of this experiment is shown in Table 2. It can be seen that a much lower number of Cm^R transductants were obtained with DM800 (34) than with EM205 (404), which reflects the diffi- culty of introducing an *rnhA* null allele within a *topA* null mutant. This is further supported by the fact that only 2 % of the DM800 Kan^R transductants are Cm^R, whereas over 50 % of the EM205 Kan^R transductants are also Cm^R. Therefore, in the trans- duction experiment, when the recipient strain is DM800, the *rnhA*::*cam* allele behaves as an excluded marker. Upon restreaking the DM800 Cm^R transductants several times in the absence of a selective pressure (LB medium without chloram- phenicol), they eventually all lost their chloram-

phenicol resistance, suggesting the presence of gene duplications which are known to be lost very frequently (Anderson & Roth, 1981). The presence of such duplications within the DM800 transduc- tants, a copy of the wild-type *rnhA* allele plus a copy of the mutated one, *rnhA*::*cam*, was confirmed by PCR experiments (data not shown). These results convincingly demonstrate that the *rnhA* gene is essential in DM800 and, moreover, suggest that duplications of this gene within this strain confer a growth advantage.

When DNA gyrase activity is increased in DM800 by substituting the *gyrB225* allele for a *gyrB*⁺ allele, RNase H overproduction is clearly shown to stimulate growth on solid (data not shown) and in liquid LB media (Figure 1). Indeed, growth in liquid media shows that RNase H over- production acts by shortening the lag phase, and therefore by allowing the strain to more rapidly adapt to fresh media (compare EM176/pSK760 with EM176/pSK762c). This has been previously observed for other *topA* null mutants under nutri- tional shift-up conditions (Drolet *et al.*, 1995). A subtle shortening of the lag phase for DM800 cells overproducing RNase H was also consistently observed (compare DM800/pSK760 with DM800/ pSK762c and data not shown). It can also be seen that DM800 *gyrB*⁺ overproducing RNase H reaches the stationary phase at a lower cell density than DM800 (Figure 1). This defect is therefore not cor- rected by overproducing RNase H or, alternatively, may rather be due to the increase in RNase H activity itself. Indeed, we have found that DM800 *gyrB*⁺ cells not overproducing RNase H eventually reach a stationary phase at a normal cell density (data not shown).

R-loop-dependent hypernegative supercoiling preferentially occurs at low temperatures

We have previously shown that RNase H-sensi- tive hypernegatively supercoiled plasmid DNA was produced when the *tetA* gene of either pBR322 or pACYC184 was transcribed but not translated in the absence of DNA topoisomerase I (Massé & Drolet, 1999b). This was accomplished by either deleting the ribosome binding site and the 5′ por- tion of the coding region of *tetA* (pBR322Δ*tet5*′ and pACYC184Δ*tet5*′), or by exposing cells carrying pBR322 to the protein synthesis inhibitor, spectino-

Table 1. Bacterial strains used in this study

Strain	Genotype	Reference or construction
DM800	Δ*(topA cysB)204*, *gyrB225*, *acrA13*	DiNardo *et al.* (1982)
EM169	DM800 *gyrB221* (Cou^R) *gyrB203* (Ts)	Massé & Drolet (1999a)
EM176	DM800 *gyrB*⁺	Massé & Drolet (1999a)
EM205	DM800 *topA*⁺, *cysB*⁺	Massé *et al.* (1997)
RFM480	*rpsL*, *galK2*, Δ*lac74*, *gyrB221* (Cou^R), *gyrB203* (Ts), *topA20*::Tn*10*	Drolet *et al.* (1995)
CAG18633	*zag-3198*::Tn*10kan*	Singer *et al.* (1989)
MIC1020	AB1157 *rnh-339*::*cat*	Itaya and Crouch (1991)
PH329	MIC1020 *zag-3198*::Tn*10kan*	MIC1020XP1.CAG18633, select Kan^R, Cm^R

Table 2. The *rnhA* gene is essential for the *topA* null mutant DM800

| Recipient | Number of transductants | | Fraction of Cm^R Kan^R transductants |
	Cm^R	Kan^R	
EM205 (DM800 *topA*$^+$)	404	70	26/50
DM800	34	352	2/95

P1 *vir* transduction lysate was prepared from strain PH329 (*zag-3198*::Tn *10kan rnh-339*::*cat*).

mycin. Moreover, the formation of R-loop-dependent hypernegative supercoiling was shown to correlate with growth inhibition of *topA* null mutants. The *topA* null strains used in these studies had a *gyrB*(Ts) allele that allows relatively good compensation at 37 °C but not at low temperatures, most likely because DNA gyrase regains activity as the temperature decreases (Drolet *et al.*, 1995). During the course of these studies, we noticed that the growth of a *gyrB*$^+$ derivative of DM800, without any known Ts mutations, was apparently sensitive to low temperatures (Massé & Drolet, 1999a and see below). We therefore considered the possibility that R-loop-dependent hypernegative supercoiling and inhibitory R-loops preferentially form at low temperatures. This would make sense if R-loop formation is the major problem in the absence of DNA topoisomerase I.

The strain DM800 and its *gyrB*$^+$ derivative carrying pACYC184Δ*tet5'* were therefore exposed to different temperatures and plasmid DNA was extracted and analyzed for the detection of hypernegatively supercoiled DNA, as described in Materials and Methods. Under these conditions the more negatively supercoiled topoisomers migrate slowly, except for the fastest migrating bands indi-

cated by the symbol (−), which represent hypernegatively supercoiled plasmid DNAs. To compare, we also used a DM800 derivative with a *gyrB*(Ts) allele (EM169). As can be seen in Figure 2, hypernegatively supercoiled plasmid DNA is detected when EM169 cells were grown at 28 °C and below (lanes 8 (28 °C) and 9 (21 °C)), and its accumulation increases as the temperature decreases (compare lane 8 with 9). This is expected since this strain carries the *gyrB*(Ts) allele. The temperature dependence of the accumulation of hypernegatively pACYC184Δ*tet5'* DNA was also observed when it was extracted from the *gyrB*$^+$ derivative of DM800 (EM176), that do not carry any known Ts compensatory mutations (compare lane 4 with 5 and 6, respectively, 37, 28 and 21 °C). In agreement with this assumption, we have shown in a previous study that the global DNA supercoiling level in this strain, and in its parent DM800, does not significantly change with temperature (Massé & Drolet, 1999a). A low amount of hypernegatively supercoiled DNA was also detected when DM800 was grown at 21 °C (lane 3). Therefore, the formation of R-loop-dependent hypernegatively supercoiled DNA due to *tetA* transcription, preferentially occurs at low temperatures and correlates
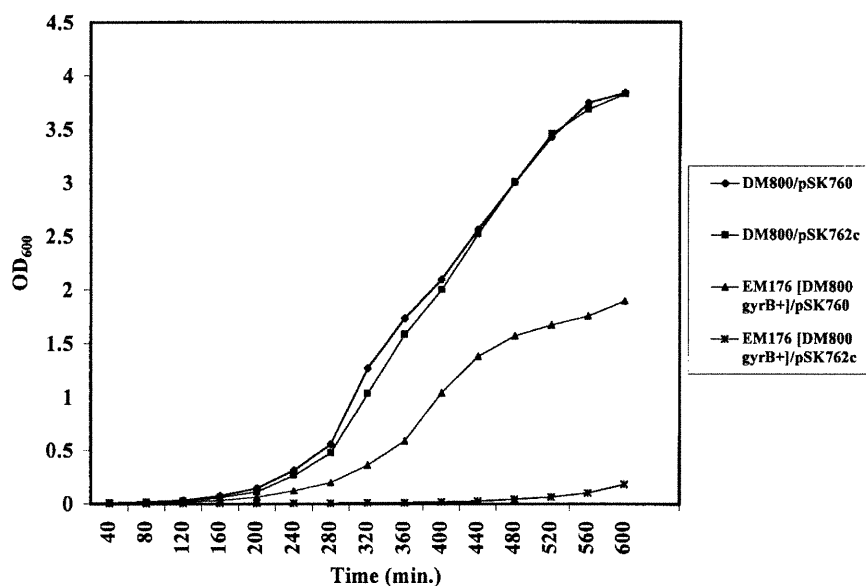

Figure 1. The effect of RNase H overproduction on the growth of DM800 and its *gyrB*$^+$ derivative in liquid medium. Fresh transformants carrying either pSK760 (*rnhA*$^+$) or pSK762c (*rnhA*$^-$) obtained at 37 °C on LB plates (+ampicillin) were grown in liquid LB medium (+ampicillin) at 37 °C.
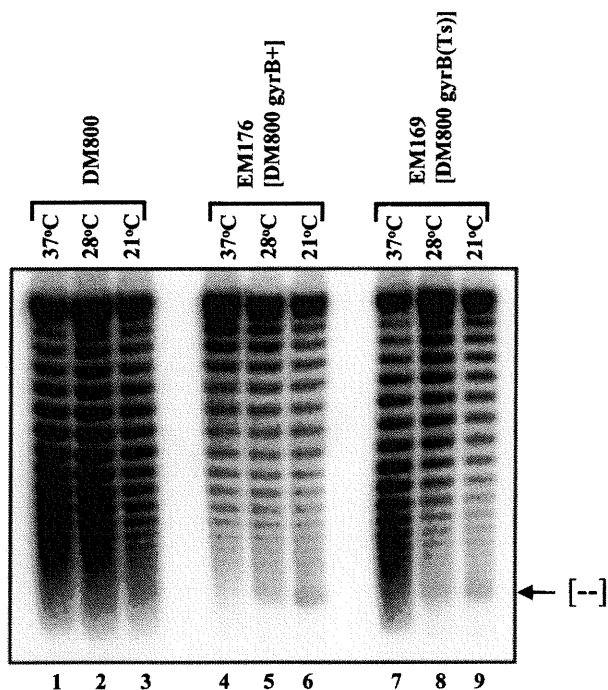
Figure 2. R-loop-dependent hypernegative supercoiling of pACYC184Δtet5′ preferentially occurs at low temperatures. Bacterial cells carrying pACYC184Δtet5′ were grown overnight in LB medium (+chloramphenicol) at 37 °C, diluted 1/75 in prewarmed LB medium (+chloramphenicol) and grown to an A_{600} of 0.4 at the same temperature. Bacterial cultures were then split and transferred to the indicated temperatures and the plasmid DNA was extracted as described in Materials and Methods. The samples were analyzed by electrophoresis in an agarose gel containing 7.5 µg/ml of chloroquine. (−) represents hypernegatively supercoiled pACYC184Δtet5′ DNA.

with the level of DNA gyrase activity. Indeed, significantly fewer such topoisomers are produced in DM800 which carries a mutated DNA gyrase (*gyrB225*) as compared to EM176 carrying a wild-type *gyrB* allele (compare lane 3 with 6). It is also interesting to note that R-loop-dependent hypernegatively supercoiled DNA was never detected when the strains, including EM176 (lane 4), were grown at 37 °C. Therefore, despite the presence of a wild-type *gyrB* allele (EM176), R-loop formation during *tetA* transcription is detectable only when the cells are exposed to low temperatures.

To further study the effect of the temperature on R-loop formation during *tetA* transcription, *topA* null strains carrying pBR322 were exposed to or not exposed to the protein synthesis inhibitor, spectinomycin. In the presence of this antibiotic, hypernegative supercoiling of pBR322 was shown to be due almost exclusively to *tetA* transcription (Massé & Drolet, 1999b). In its absence, anchoring of the TetA protein to the membrane during coupled transcription-translation, was shown to be responsible for the formation of hypernegatively supercoiled pBR322 DNA (Lynch & Wang, 1993).

Under such conditions the rotation of the RNA polymerase around the DNA is completely inhibited and this leads to the generation of very high level of supercoiling (the twin-domain model; Liu & Wang, 1987). Therefore, our experiments also allow to compare R-loop-dependent with translation-dependent hypernegative supercoiling, as a function of temperature.

We first performed a kinetic experiment to follow the accumulation of hypernegatively supercoiled pBR322 following a temperature downshift of DM800 cells from 37 to 21 °C, and in the presence or in the absence of spectinomycin (Figure 3). It can be seen that translation-independent (R-loop-dependent) hypernegatively supercoiled pBR322 slowly accumulates with time (lanes 1 to 3). Similar observations were made when R-loop-dependent hypernegative supercoiling was detected by extracting other plasmid DNAs (pBR322Δtet5′ and pACYC184Δtet5′) from several *topA* null mutants exposed to the same temperature (Massé & Drolet, 1999b and data not shown). Translation-dependent hypernegatively supercoiled pBR322 also accumulates in parallel with R-loop-dependent hypernegatively supercoiled DNA (lanes 4 to 6). Not only do both types of such
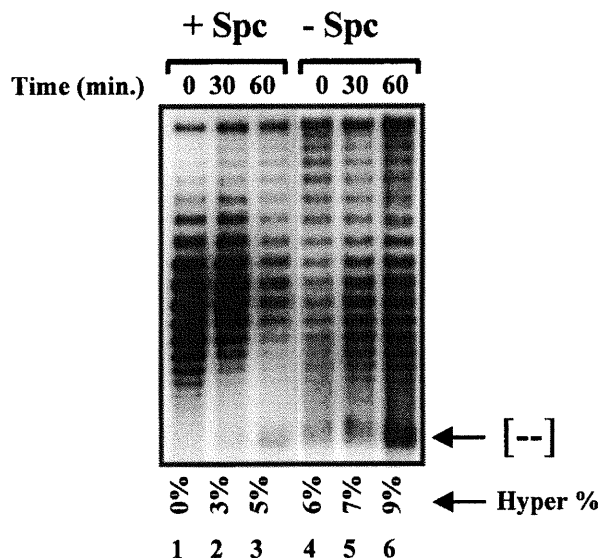
Figure 3. R-loop-dependent hypernegatively supercoiled pBR322 DNA slowly accumulates with time in DM800 cells exposed to 21 °C. DM800 cells carrying pBR322 were grown overnight in LB medium (+ampicillin) at 37 °C, diluted 1/75 in prewarmed LB medium (+ampicillin) and grown to an A_{600} of 0.4 at the same temperature. They were treated (+Spc) or not (−Spc) with spectinomycin for 15 minutes and then transferred to 21 °C. Plasmid DNA was extracted at the indicated time as described in Materials and Methods. The samples were analyzed by electrophoresis in an agarose gel containing 7.5 µg/ml of chloroquine. (−) Represents hypernegatively supercoiled pBR322 DNA. Hyper % reflects the percentage of hypernegatively supercoiled DNA in the total population of plasmid topoisomers as measured by densitometry.

topoisomers accumulate in parallel with each other, but they also increase roughly by the same proportion with time, as measured by densitometry (for example, compare lanes 2 and 3 with lanes 5 and 6). This may suggest that the newly synthesized hypernegatively supercoiled pBR322 in the absence of spectinomycin, also reflects R-loop-dependent supercoiling (see below). It is also interesting to note that R-loop-dependent hypernegative supercoiling is produced in DM800 cells at 21 °C, despite the fact that the global supercoiling level in this strain is lower than in a wild-type cell. This supports our previous conclusion that R-loop formation involves local rather than global supercoiling. By using the transcription inhibitor rifampicin, we also found that hypernegatively supercoiled pBR322 generated at 21 °C slowly disappears with time (data not shown). However, the turnover of such topoisomers at 28 °C is more rapid and similar to the turnover of translation-dependent hypernegative supercoiling at the same temperature (data not shown).

We next extracted pBR322 DNA from *topA* null mutants with different growth capabilities after an exposition to various temperatures, in the presence or in the absence of spectinomycin (Figure 4). First of all, we found that translation-dependent hypernegative supercoiling of pBR322 at 37 °C does not correlate either with DNA gyrase activity or with the growth defect of the *topA* null strain from which the DNA is extracted. Indeed, it can be seen that roughly the same amount of hypernegatively supercoiled pBR322 is produced in DM800 and its *gyrB*+ derivative, EM176 (compare lane 4 with lane 10), at 37 °C, despite the fact that DM800 grows much better (Figure 1) and carries a mutated DNA gyrase. Moreover, the formation of such topoisomers in RFM480 cells grown at 37 °C is barely detectable (lane 16), despite the fact that this strain is sicker than DM800 and requires RNase H overproduction for optimal growth. However, the formation of hypernegatively supercoiled pBR322 in the absence of spectinomycin correlates with the growth defect of the *topA* null strain, when the cells are exposed to lower temperatures (28 °C and 21 °C). Indeed, it can be seen that a larger proportion of such topoisomers are produced from RFM480 exposed to 28 °C and 21 °C as compared to DM800, with EM176 being in the middle range but closer to RFM480 (compare lane 5 with 11 and 17, 28 °C and lane 6 with 12 and 18, 21 °C). This matches very well with the known growth defect of these strains (Massé & Drolet, 1999a) and leads us to believe that an important proportion of hypernegatively supercoiled pBR322 produced at lower temperatures, reflects R-loop-dependent supercoiling. In fact, R-loop-dependent hypernegative supercoiling, as revealed following exposition of the strains to spectinomycin, perfectly correlates with the growth defect of the *topA* null strains (compare lane 2 with 8 and 14, 28 °C and lane 3 with 9 and 15, 21 °C). Note that, in agreement with the results of previous studies (Massé & Drolet,

1999a,b; see Figure 2), such topoisomers were never detected in strains grown at 37 °C (lanes 1, 7 and 13). As noted above (Figure 3), we also found that an increase in the amount of R-loop-dependent hypernegatively supercoiled DNA due to an exposition of the strains to lower temperatures, is also accompanied by a similar increase in the amount of "translation-dependent" hypernegative supercoiling (for example, compare lane 1 with 2 and lane 4 with 5). This supports our belief that R-loop formation during *tetA* transcription can occur at lower temperatures, despite the fact that *tetA* mRNA is translated. However, as noted before (Massé & Drolet, 1999b), translation can also be inhibitory, as suggested by the fact that R-loop-dependent hypernegative supercoiling is sometimes generated in a larger proportion than "translation-dependent" hypernegative supercoiling. This is observed for the strain showing the most severe growth defect and the highest requirement for RNase H overproduction, RFM480 (compare lane 14 with 17, 28 °C). A hallmark of translation-dependent supercoiling in the frame of the twin-domain model, is the generation of a heterogeneous distribution of topoisomers (see, for example, Pruss & Drlica, 1986; Wu *et al.*, 1988; Lynch *et al.*, 1993), as also shown here when the cells were grown at 37 °C in the absence of spectinomycin (lanes 4, 10 and 16). However, we note that this heterogeneity is lost as the temperature decreases (for example, compare lane 4 with 6 or 16 with 18). This absence of heterogeneity was previously observed when hypernegative supercoiling was R-loop-dependent (Massé & Drolet, 1999b), and is also observed in the present study when R-loop formation is involved (for example, compare lane 2 with 4; see Discussion).

The fact that *tetA* is the key gene for the generation of hypernegatively supercoiled DNA both in the presence and in the absence of spectinomycin, is certainly more than a coincidence. It supports our conclusion that R-loop is also involved in hypernegative supercoiling of pBR322 at lower temperatures, despite the fact that translation is still going on. In agreement with this conclusion, is our observation that tetracycline resistance of DM800 cells carrying pBR322 at low temperatures, decreases when RNase H is overproduced (data not shown). In this case, RNase H overproduction is inhibitory, presumably because *tetA* mRNA, in the R-loop, is degraded before being freed from the R-loop by other processes that do not involve RNA degradation (see below). Therefore, a careful kinetic analysis could reveal a partial sensitivity of hypernegative supercoiling of pBR322 to RNase H overproduction at lower temperatures. Moreover, according to the twin-domain model for transcription, the generation of hypernegatively supercoiled plasmid DNA should be disfavored at lower temperatures because of lower transcription velocities. Under these conditions, the diffusion of supercoiling domains should be favored over their accumulation.

EM176
[DM800 *gyrB*+]   **DM800**   **RFM480**

| + Spc | - Spc | + Spc | - Spc | + Spc | - Spc |

37°C 28°C 21°C | 37°C 28°C 21°C | 37°C 28°C 21°C | 37°C 28°C 21°C | 37°C 28°C 21°C | 37°C 28°C 21°C

0% 6% 11% 2% 11% 20% | 0% 3% 8% 4% 7% 10% | 0% 28% 21% 0% 14% 28%

1 2 3 4 5 6   7 8 9 10 11 12   13 14 15 16 17 18

← [--]

← Hyper %

Figure 4. Hypernegative super-coiling of pBR322 at low temperatures correlates with the growth defect of *topA* null mutants. Bacterial cells carrying pBR322 were grown overnight in LB medium (+ampicillin) at 37 °C, diluted 1/75 in prewarmed LB medium (+ampicillin) and grown to an A_{600} of 0.4 at the same temperature. Bacterial cultures were treated (+Spc) or not (−Spc) with spectinomycin for 15 minutes and then split and transferred to the indicated temperatures and incubated for 30 minutes at 37 °C or 60 minutes at 28 and 21 °C. Plasmid DNA was then extracted as described in Materials and Methods. The samples were analyzed by electrophoresis in an agarose gel containing 7.5 µg/ml of chloroquine. (−) Represents hypernegatively super-coiled pBR322 DNA. Hyper % reflects the percentage of hypernegatively supercoiled DNA in the total population of plasmid topoisomers as measured by densitometry.

The growth problem of *E. coli topA* null mutants is exacerbated at low temperatures

Since R-loop formation is a major problem in the absence of DNA topoisomerase I and seems to occur preferentially at low temperatures, the growth of *topA* null mutants should be sensitive to such temperature drops. Moreover, these growth conditions should affect *topA* null mutants that benefit from the presence of appropriate compensatory mutations less than the ones suffering from the absence of such mutations. Comparing the growth capability of DM800 and its *gyrB+* derivative (EM176) as a function of temperature directly tested this. As predicted, we found that the appearance of visible colonies of EM176 is much more delayed by low temperatures than colonies of DM800 (data not shown). Colonies of EM176 are visible at 28 and 21 °C only after prolonged periods of incubation.

We next studied the effect of overproducing RNase H on the growth of these strains at low temperatures. By looking at the colony size, we first noted that overproducing RNase H has a negative effect on the growth of DM800 at 21 °C, whereas it has no noticeable effect at 37 °C (Figure 5). We found that RNase H overproduction significantly stimulates the growth of EM176 at 37 °C (Figure 1) but has a clear negative effect at 21 °C. Indeed, this effect is more dramatic for EM176 than its parent DM800, since a clear drop in the efficiency of plating (EOP) is observed for EM176 but not DM800 (Table 3 and data not shown). In the case of DM800, only the colony size as shown above, but not the number of colonies, is affected. RNase H overproduction reduces the viability of EM176 at 21 °C by more than four orders of magnitude (Table 3, EM176/pSK760). A fivefold

drop in the viability is also observed when RNase H is not overproduced (Table 3, EM176/pSK762c). Again, these results perfectly correlate with the growth defect of these strains. As expected, the negative effect of RNase H overproduction at 21 °C is enhanced by the presence of a DNA gyrase that is more active in the promotion of R-loop elongation (EM176). This negative effect of RNase H is illustrated by the fact that tetracycline resistance from pBR322 is reduced in the absence of DNA topoisomerase I, when RNase H is overproduced at low temperatures (see above). Presumably, some R-loops must be removed by processes that do not involve RNA degradation, in order for the mRNA to be available for protein synthesis. Our recent experimental evidence strongly suggests that one of these alternative processes involves the participation of DNA topoisomerase III, which is able to act as soon as the R-loop is initiated to destabilize it (Broccoli *et al.*, 1999).

Discussion

Here, we have shown that R-loop formation could be problematic in the *topA* null mutant DM800, in which the absence of DNA topoisomerase I has been very well compensated for by naturally selected mutations (DiNardo *et al.*, 1982). This is observed despite the fact that the global level of chromosomal negative supercoiling is below the wild-type level in this strain (Pruss *et al.*, 1982). This further supports our previous conclusion that local rather than global supercoiling is linked to R-loop formation and the key role played by DNA topoisomerase I (Massé & Drolet, 1999b). More importantly, this result highlights the major role played by DNA topoisomerase I in the inhibition
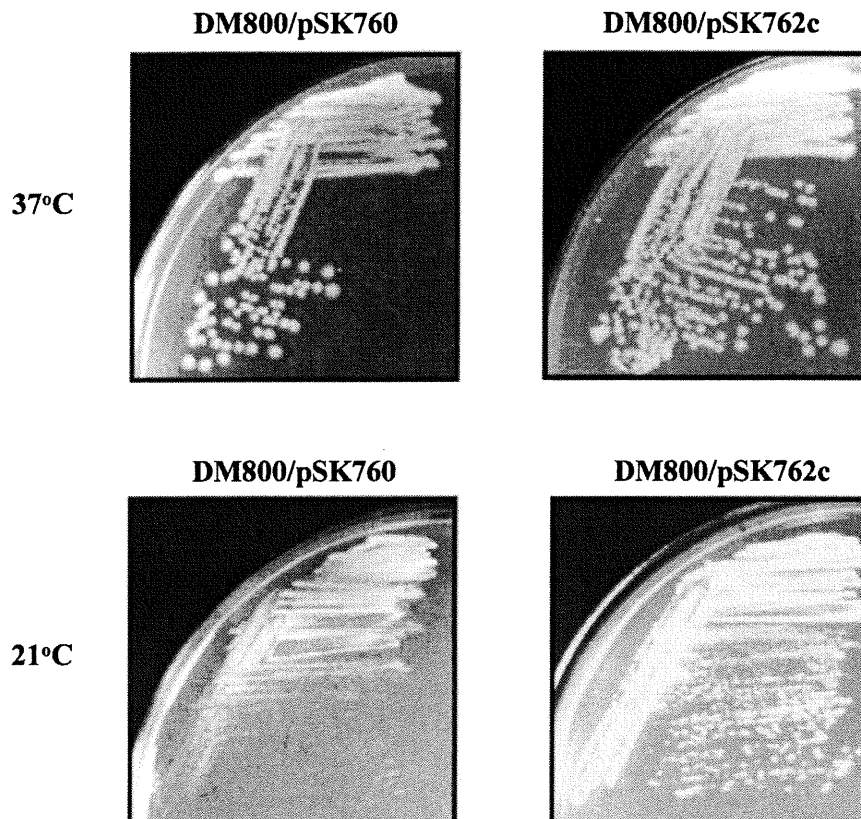
DM800/pSK760   **DM800/pSK762c**

37°C

DM800/pSK760   **DM800/pSK762c**

21°C

Figure 5. RNase H overproduction negatively affects the growth of DM800 at 21 °C. Fresh transformants of DM800 carrying either pSK760 (*rnhA*⁺) or pSK762c (*rnhA*⁻) obtained at 37 °C on LB plates (+ampicillin) were streaked for single colonies on LB plates (+ampicillin) and incubated at 21 and 37 °C, respectively, for 52 and 22 hours.

of R-loop formation during transcription at the optimal growth temperature for *E. coli*, since the transduction experiments were performed at this temperature (37 °C). Moreover, for the first time we were able to show that RNase H overproduction in *topA* null mutants is required for good growth at 37 °C, when DNA gyrase is very active (strain EM176).

Despite the fact that our results clearly show that R-loop formation can occur and be problematic for growth at 37 °C, they also reveal that it preferentially occurs at lower and suboptimal temperatures. This is first illustrated by the fact that significant and detectable R-loop formation during *tetA* transcription on pACYC184 and pBR322 only

occurs at low temperatures. Second, as expected, the growth problem of *topA* null mutants is exacerbated at low temperatures in a manner that is linked, at least in part, to R-loop formation. This is found irrespective of the presence of Ts compensatory mutations. Interestingly, GyrA has been previously identified as a cold-shock protein and the synthesis of GyrB was also shown to be sustained during the cold-shock response (Jones *et al.*, 1992b). Therefore, an increase in the amount of DNA gyrase per cell could explain both the sensitivity of *topA* null mutants to low temperatures and the increase in R-loop formation under these conditions. However, we consider this possibility rather unlikely for the following reasons. First, a downshift of 13 deg. C or more is required to induce the cold-shock response, and the magnitude of this response increases with the range of the temperature shift (Jones *et al.*, 1992a; Jones & Inouye, 1994). A large amount of R-loop-dependent hypernegatively supercoiled DNA is produced when *topA* null cells are transferred from 37 to 28 °C (a 9 deg. C downshift; see for example, Figure 4). Second, before the increase in the GyrA synthesis rate following a temperature downshift to induce the cold-shock response, there is a lag period of almost one hour during which the GyrA synthesis rate actually decreases (Jones *et al.*, 1992b). R-loop-dependent hypernegatively super-

Table 3. Overproduction of RNase H inhibits the gowth of EM176 (DM800 *gyrB*⁺) at 21 °C

Strains	Ratio of colonies at 21 °C to colonies at 37 °C
EM176/pSK760	5.5×10^{-5}
EM176/pSK762c	0.2

Very tiny colonies of fresh transformants obtained at 37 °C on LB (+ampicillin) were resuspended in liquid LB medium to give a cell density similar to a culture grown to saturation. Cells were diluted and plated on LB (+ampicillin). Incubations at 37 and 21 °C were for 48 hours and ten days, respectively.

coiled DNA is detected in large amounts at least within ten and 30 minutes following transfers of *topA* null mutants, respectively, from 37 to 28 °C and 37 to 21 °C (data not shown). Most of all, this accumulation of R-loop-dependent hypernegatively supercoiled DNA at low temperatures is observed in the presence of the protein synthesis inhibitor spectinomycin, that is added before the temperature downshifts, therefore precluding *de novo* synthesis of DNA gyrase. Another hypothesis, the one we favor, to explain both the cold-sensitivity of *topA* null mutants and the increase in R-loop formation at low temperatures, is based on the reduced transcription velocity that immediately follows temperature downshifts. Indeed, our previous results have strongly suggested that R-loop formation is initiated by the reannealing of the nascent RNA with the complementary DNA template region behind the moving RNA polymerase (Massé & Drolet, 1999b). In this context, the effect of the temperature on R-loop formation can be explained by a reduced transcription velocity at low temperatures that favors R-loop initiation. In fact, this hypothesis is supported by the results of *in vitro* transcription experiments that were not originally designed to test it (Daube & von Hippel, 1994). In this series of experiments, Daube & von Hippel studied 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 the template DNA strand. Proper RNA displacement from the RNA polymerase was shown to occur normally during transcription initiated in this way, with either *E. coli* or phage T7 RNA polymerase. However, when the transcription velocity was reduced, the actively displaced nascent RNA was shown to reanneal with the template DNA strand within the permanent bubble. Reduction of the transcription velocity was accomplished by either using *E. coli* RNA polymerase instead of phage T7 RNA polymerase, or by reducing the concentration of nucleotides during transcription with T7 RNA polymerase. This result directly demonstrates that lower transcription velocities promote R-loop initiation. Also note that the presence of a permanently unwounded DNA region behind a moving RNA polymerase can be considered as an optimal condition for R-loop initiation in the frame of our model (Massé & Drolet, 1999b). This was indeed shown to occur in these experiments. However, an important factor that is not manifested in *in vitro* transcription experiments, but must be considered *in vivo*, is the fact that transcription and translation are tightly coupled in bacteria. In this context, translation, and hence the binding of ribosomes to the nascent RNA, is expected to render this RNA unavailable for reannealing with the complemen-

tary DNA template region behind the moving RNA polymerase. This was indeed recently shown to be the case (Massé & Drolet, 1999b). Therefore, in addition to the reduced transcription velocity at lower temperatures, a weaker coupling of translation and transcription must also be evoked to explain the increased propensity to R-loop formation under such growth conditions. On the contrary, a reduced propensity to R-loop formation during transcription at 37 °C is explained by both an increased transcription velocity and a tight coupling of translation and transcription.

Our current model for R-loop formation during transcription in *Escherichia coli* is schematically represented in Figure 6. In the first situation (1), the tight coupling of translation and transcription inhibits R-loop formation. In the second situation (2), a weak coupling of translation and transcription leads to R-loop formation when transcription-induced negative supercoiling is not relaxed by DNA topoisomerase I. In the third situation (3), the absence of translation allows R-loop formation. This occurs when the transcribed genes encode for an untranslated RNA or when translation of mRNAs is inhibited due to the use of appropriate antibiotics or under certain growth conditions (see below). Under such conditions, when the *tetA* gene of pBR322 is transcribed at low temperatures, a bimodal distribution of topoisomers is generated. One population of topoisomers reflects the global supercoiling level, whereas the other population is hypernegatively supercoiled, and reflects R-loop formation. This bimodal distribution can be explained in the following way. Not every transcriptional event of *tetA* will lead to R-loop initiation. If R-loop is not initiated, or if RNase H acts as soon as it is initiated, there will be no significant changes in plasmid supercoiling level, which will therefore reflects global supercoiling level. When *tetA* mRNA is translated, every transcriptional event will generate positive supercoiling, that will either diffuse or immediately be relaxed by DNA gyrase before its diffusion. This will result in a heterogeneous distribution of topoisomers. When R-loop formation is initiated and RNase H does not act rapidly enough, the following scenario could be imagined (Figure 6, (4) to (5)). Since a bimodal distribution of topoisomers is generated, we can speculate that when R-loop formation is initiated it elongates enough without interruption to generate hypernegatively supercoiled DNA. This bimodal distribution of topoisomers was also observed when the formation of hypernegatively supercoiled DNA was reduced by overproducing RNase H (Massé & Drolet, 1999b). We interpret these results as indicating that R-loops trigger the formation of such topoisomers, but are only partially responsible for their stabilization. One possibility is that once R-loop formation is initiated, RecA or single-strand binding (SSB) proteins bind to the single-stranded DNA of the R-loop. The binding of RecA or SSB progresses with R-loop elongation (Figure 6, (5)). Therefore,
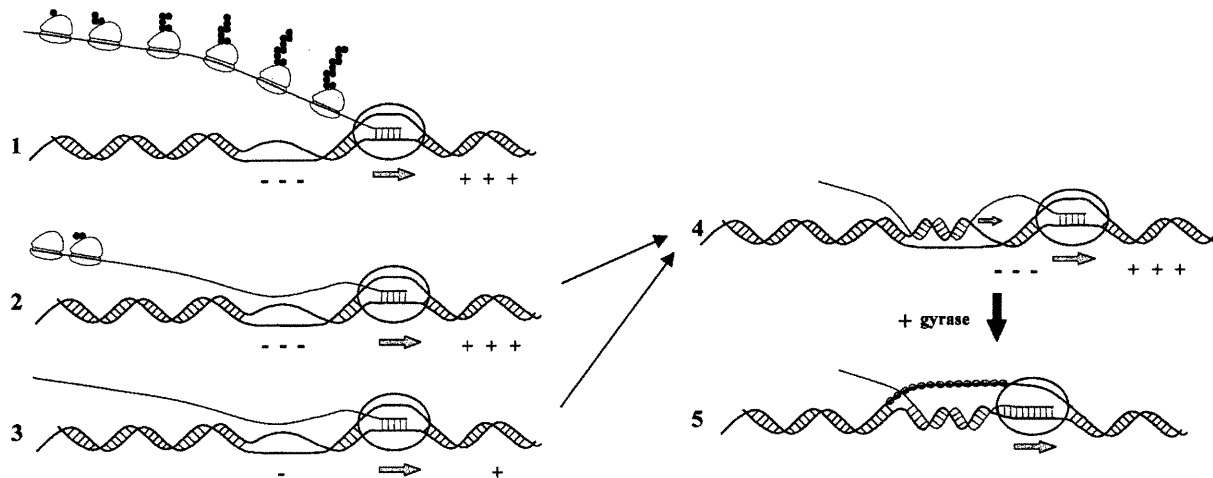
Figure 6. A model for R-loop formation in *Escherichia coli*. See the text for details. − and + represents negative and positive supercoiling, respectively. In (5), the small circles covering single-strand DNA represents SSB protein or RecA.

even if RNase H degrades the RNA within the R-loop, the DNA will stay unwound and hypernegative supercoiling will be revealed following the denaturing of proteins during DNA extraction. Hypernegative supercoiling can be generated because DNA gyrase reintroduces the negative supercoils that are lost in the process of R-loop elongation. DNA gyrase can also directly contributes to this process by relaxing the positive supercoiling in front of the moving RNA polymerase. However, the properties of DNA gyrase that are required for R-loop formation are different, at least in part, from the ones required to generate hypernegatively supercoiled DNA during coupled transcription-translation of *tetA*, in the frame of the twin-domain model. Indeed, we found that neither the growth defect of *topA* null mutants nor R-loop formation correlate with the ability of DNA gyrase to produce translation-dependent hypernegatively supercoiled pBR322 DNA (Figure 4). Presumably, R-loop formation requires both the negative supercoiling activity of DNA gyrase and its ability to relax the positive supercoiling generated during transcription. Interestingly, hypernegative supercoiling of plasmid DNA has been shown to occur *in vitro* when both DNA gyrase and SSB proteins are incubated in the presence of the preprimosome that possesses a helicase activity (Parada & Marians, 1989).

The fact that DNA topoisomerase I might be required for the cells to adapt to suboptimal temperatures has also been previously suggested. In these studies, the effects of high temperatures were evaluated, whereas in the present work we have demonstrated the sensitivity of *topA* null mutants to low temperatures. It was shown that one of the promoters of the *topA* gene is a heat-shock promoter (Lesley *et al.*, 1990). A strain carrying a deletion of this σ^{32}-promoter was shown to be sensitive to high temperatures (Qi *et al.*, 1996). Interestingly,

much experimental evidence suggests that ribosomes are prokaryotic sensors for the heat and cold shock response networks (VanBogelen & Neidhardt, 1990; Jones & Inouye, 1996). Treatment with ethanol (VanBogelen *et al.*, 1987) and depletion of 4.5 S RNA (Bourgaize *et al.*, 1990) alter the translational capacity of the cell and induce the heat-shock response. Early studies have demonstrated a transient inhibition of protein synthesis upon shifting *E. coli* cells from 36 °C to 28 °C (Lemaux *et al.*, 1978). We propose that R-loop formation is more prone to occur during temperature shifts because of a transient inhibition of translation and, therefore, a transient decoupling of translation and transcription (Figure 6, (2) and (3)). DNA topoisomerase I will thus be required under such conditions to repress R-loop formation that may otherwise inhibit the expression of a set of genes required for adaptation. Interestingly, a stationary phase promoter (σ^s) was recently found for the *topA* gene of *E. coli* (Qi *et al.*, 1997). A decrease in the overall translational activity is known to occur concomitantly with the transition from the exponential growth phase to the stationary phase of *E. coli* cells. This is accompanied by the appearance of inactive 100 S ribosomes, which are dimers of 70 S ribosomes (Wada *et al.*, 1990). In the present work we showed that the entry of a *topA* null mutant to the stationary phase could be sooner than usual and in a manner linked to R-loop formation (Figure 1). DNA topoisomerase I may therefore be required for the entry of the cells to the stationary phase for the same reasons that explain its requirement during temperature shifts. The need for DNA topoisomerase I activity during the lag phase of growth (Figure 1) and during nutritional shift-ups (Drolet *et al.*, 1995) may also be explained in the same way. In fact, a transient reduction in the translational capacity is known to occur with a transfer of the cells from old to fresh

media (see, for example, Champney, 1977). In conclusion, the current experimental data allow us to propose that DNA topoisomerase I is required to inhibit R-loop formation due to the uncoupling of translation and transcription that occurs when the cells are exposed to various changes in growth conditions.

Materials and Methods

E. coli strains

The *E. coli* strains used are listed and described in Table 1. When indicated, they were constructed by transduction using P1*vir* as described (Miller, 1992). Because EM176 (DM800 *gyrB*$^+$) cells tend to accumulate *topA* compensatory mutation(s) at a very high frequency, tiny and barely visible fresh colonies were always used in the experiments involving this strain.

Plasmids

pACYC184Δ*tet5'* is a pACYC184 derivative in which the 5' portion of the *tetA* gene, including the ribosome binding site and the 5' portion of TetA has been deleted by an *Eco*RV-*Hind*III deletion (Massé & Drolet, 1999a,b). A new −10 sequence for the *tetA* promoter was created as a result of this deletion. pSK760 and pSK762c are pBR322 derivatives that, respectively, carry the wild-type *rnhA* gene or a mutated and inactive version of this gene (Drolet et al., 1995).

Media and growth conditions

Unless otherwise indicated, bacteria were grown in LB medium supplemented with 50 μg/ml cysteine at the temperature indicated in the Table and Figure legends. When needed, antibiotics were added as follows: ampicillin and kanamycin at 50 μg/ml and chloramphenicol at 30 μg/ml. Because of the *acrA13* mutation in the DM800 derivatives that renders these cells more permeable to many antibiotics (DiNardo et al., 1982), chloramphenicol and kanamycin were used at 10 and 15 μg/ml, respectively, for these cells. When indicated, spectinomycin was used at 400 μg/ml to inhibit protein synthesis.

DNA supercoiling analysis

For the extraction of plasmid DNAs for supercoiling analysis, bacterial cells were grown overnight in LB medium supplemented with cysteine at 37 °C and then diluted 1/75 in pre-warmed medium. The cells were grown to an A_{600} of 0.4 at 37 °C at which time they were transferred to the desired temperature. The plasmid DNAs were extracted when the A_{600} reached about 0.7, or, unless otherwise indicated, after an exposition of about two hours at the respective temperature, when an A_{600} of 0.7 could not be reached. When indicated, the cells were exposed to spectinomycin at 37 °C for 15 minutes before being transferred to the indicated temperature. Growth was stopped by transferring the cells in a tube filled with ice. By this procedure, the temperature of the cultures immediately dropped to 0 °C. Plasmid DNAs were extracted by an alkaline lysis procedure as described (Massé & Drolet, 1999a).

Electrophoresis in the presence of chloroquine

One-dimensional agarose gel electrophoresis in the presence of chloroquine at 7.5 μg/ml in 0.5 × TBE was performed essentially as described (Phoenix et al., 1997). After electrophoresis, agarose gels were dried and prepared for *in situ* hybridization as performed before (Phoenix et al., 1997).

Current molecular biology techniques

The current molecular biology techniques were performed essentially as described (Sambrook et al., 1989). CaCl$_2$ transformations were carried out as described (Drolet et al., 1995).

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8 Discussion

Le modèle classique de régulation du surenroulement de l'ADN chez les cellules procaryotes propose un équilibre entre l'introduction de supertours négatifs par la gyrase et la relaxation de ceux-ci catalysée par la topoisomérase I (Drlica, 1992). Ce modèle est basé sur les résultats de DiNardo *et al.* (1982) qui ont démontré que la perte de la topoisomérase I chez *E. coli* induisait l'apparition de mutations compensatoires. Ces mutations se trouvaient, le plus souvent, dans les gènes codant pour la gyrase et provoquaient une diminution de l'activité de l'enzyme. L'interprétation des auteurs favorisait l'hypothèse qu'en absence de topoisomérase I, le surenroulement de l'ADN devient probablement trop élevé pour permettre la croissance des cellules. Dans ce contexte, une diminution de l'activité de la gyrase permettrait de retrouver un surenroulement global de l'ADN plus compatible avec la viabilité de la bactérie.

Depuis, de nouvelles données *in vitro* et *in vivo* ont permis une interprétation différente du rôle de la topoisomérase I chez *Escherichia coli*. Les expériences de transcription *in vitro*, en absence de la topoisomérase I et en présence de la gyrase, démontrèrent la formation de R-loops durant la transcription par la polymérase ARN bactérienne (Drolet *et al.*, 1994a). À partir de ces résultats, un modèle expliquant la formation des hybrides ARN-ADN fut proposé. Celui-ci suggérait que l'activité de la gyrase favorisait la formation de ces structures alors que la topoisomérase I et la RNase H en inhibaient la formation. Par la suite, les travaux *in vivo* suggérèrent que chez les mutants *topA* de *E. coli* où la gyrase est très active, la formation d'hybrides ARN-ADN était particulièrement favorisée. Donc, le modèle proposé de formation de R-loops se montrait tout aussi valable afin d'expliquer les données obtenues *in vivo* et *in vitro*. De plus, les hybrides semblaient directement responsables du faible taux de croissance observé chez les mutants *topA*, puisque l'élimination de ces structures par la surproduction intracellulaire de la RNase H aidait significativement la croissance des mutants *topA* (Drolet *et al.*, 1995).

Donc, la formation de R-loops semblait être un facteur important de l'inhibition de croissance des mutants *topA*. Cependant, peu d'information existait, à ce moment, quant à leur fréquence ou leurs sites de formation. À ce sujet, plusieurs caractéristiques spécifiques aux opérons ribosomaux favorisaient particulièrement ces gènes comme pouvant représenter des sites potentiels de formation de R-loops. Les résultats ont démontré que la transcription de l'opéron *rrnB* incomplet, porté sur un vecteur plasmidique, provoque une inhibition de croissance cellulaire chez les mutants *topA*. L'arrêt de croissance semble directement relié à la formation d'hybrides ARN-ADN puisque ce n'est que lorsque la RNase H est surproduite que les mutants *topA* peuvent croître. Dans le but de vérifier directement la présence de R-loops sur les matrices transcrites, nous avons utilisé une technique d'extraction non-dénaturante de l'ADN afin de ne pas induire la formation de R-loops sur les plasmides surenroulés négativement (Richardson, 1975). Il a été montré que la transcription d'un fragment *Hin*dIII de 567 pb provenant de la région 16S de l'opéron *rrnB* provoquait l'accumulation de conformères. Malgré l'utilisation d'une technique d'extraction de l'ADN non-dénaturante, il aurait pu être possible d'observer un effet relié à un artefact. Cette hypothèse a été écartée par les résultats de la surproduction *in vivo* de la RNase H, qui démontrent une diminution importante de la quantité de conformères relativement au témoin où la RNase H n'était pas surproduite. De plus, l'analyse topologique de ces constructions démontre une proportion importante de plasmides hypernégativement surenroulés lorsque la gyrase est très active. La présence des topoisomères hypernégatifs s'explique par la formation de R-loops provoquant l'introduction de supertours négatifs supplémentaires par la gyrase. Ceci explique d'ailleurs pourquoi, lorsque la RNase H est surproduite, on observe une diminution importante de la formation de ces topoisomères.

Les résultats de Phoenix *et al.* (1997) démontrent que la transcription *in vitro* du fragment *Hin*dIII en présence de la gyrase provoque la formation de plasmides hypernégativement surenroulés sans spécificité d'orientation du fragment. Ce n'est seulement qu'en présence de la RNase A, qui dégrade l'ARN simple-brin, que la spécificité d'orientation est observée. Aussi, il fut observé que dans un mutant *topA*, la formation de ce type de topoisomères était indépendante de l'orientation de

transcription du fragment *Hind*III (Massé *et al.*, 1997). Cependant, l'analyse de conformères démontre qu'il existe toujours un nombre supérieur de R-loops formés dans l'orientation physiologique. Il est probable qu'un nombre semblable de R-loops soit formé dans les deux orientations du fragment *Hind*III, mais que pour différentes raisons, ceux-ci soient plus stables dans l'orientation physiologique. Il est vraisemblable qu'en présence de la gyrase, la formation d'un hybride, stable ou non, provoque l'introduction rapide de supertours négatifs conduisant à l'accumulation de topoisomères hypersurenroulés négativement. L'analyse des conformères permettrait donc de mieux discriminer parmi la stabilité des hybrides formés.

Cette stabilité supérieure des R-loops spécifique à l'orientation peut s'expliquer par la séquence nucléotidique du brin matrice. Nous avons vu que les brins ARN riches en purines avaient la faculté de stabiliser la structure ARN-ADN (Roberts and Crothers, 1992). Dans le cas du fragment *Hind*III de 567 pb, le brin ARN transcrit dans l'orientation physiologique possède une plus grande concentration de purine que le brin inverse. Cette hypothèse se trouve aussi appuyée par les résultats d'études qui montrent que de longs segments riches en purine dans l'ARN favorisent la formation de R-loop (Reaban *et al.*, 1994; Grabczyk and Fishman, 1995).

Les données de transcription *in vitro* par les polymérases phagiques suggèrent aussi que durant l'élongation du R-loop, du moins dans l'orientation physiologique, l'ARN naissant n'est jamais déplacé de la matrice. En effet, la RNase A n'abolit pas sa formation (Phoenix *et al.*, 1997; Massé *et al.*, 1997). L'élongation de l'hybride, relativement à l'ARN, doit donc se faire du coté 5' vers 3' et non de 3' vers 5'. Il est intéressant de noter qu'en utilisant ce système de transcription *in vitro*, aucun R-loop ne fut détecté sur des matrices relaxées (Massé *et al.*, 1997). Une autre étude *in vitro* permit de démontrer que la formation de R-loop sur le fragment *Hind*III orienté de manière physiologique ne commençait à être détectée que lorsque le surenroulement était équivalent ou supérieur à celui que l'on retrouve dans une souche sauvage (E. Massé et M. Drolet, résultats non publiés).

Lorsque les cellules *topA gyrB*(Ts) sont incubées à 35°C, la formation de R-loops sur le fragment inversé n'est pratiquement pas détecté. À cette température, le

surenroulement global de l'ADN est plus relaxé que celui des cellules sauvages, il est donc probable qu'à cette densité de surenroulement, les hybrides ne soient pas assez stables pour être observés. D'ailleurs, l'incubation à 30°C augmente la densité de surenroulement de l'ADN, et on peut alors observer la présence de conformères sur le fragment inversé. Il est possible que les R-loops formés dans l'orientation physiologique soient moins sensibles à la densité de surenroulement, ce qui expliquerait leur présence en grand nombre, que ce soit à 35°C ou 30°C. De plus, il est intéressant de remarquer qu'aux températures d'incubation supérieures à 37.5°C, aucun conformère n'est détecté, peu importe l'orientation. À cette température, l'ADN des souches *topA gyrB*(Ts) se montre particulièrement relaxé. L'activité de la gyrase à cette température semble trop faible pour promouvoir la production de R-loops. Ceci démontre donc l'implication directe de la gyrase dans la formation des hybrides ARN-ADN.

La formation de R-loops dans les mutants *topA* est-elle uniquement spécifique à la transcription du fragment *Hind*III? Des résultats ont montré que la transcription de différents segments de l'opéron *rrnB* de même que des fragments provenant d'une banque génomique du chromosome de *E. coli*, provoquaient aussi un effet néfaste sur la croissance d'une souche *topA*, et ce, de manière dépendante du niveau intracellulaire de RNase H (E. Massé, M. Drolet, résultats non publiés).

Dans les autres études sur la formation d'hybrides ARN-ADN, l'unique rôle prévu de l'ARN nouvellement transcrit est de s'apparier avec la matrice. Ces hybrides peuvent alors servir d'amorces pour initier la réplication (Itoh and Tomizawa, 1980; Xu and Clayton, 1995) ou stimuler la recombinaison dans la commutation de classes des immunoglobulines (Reaban *et al.*, 1994; Daniels and Lieber, 1995). Il est évident que la formation des R-loops n'est pas le but ultime de la transcription des opérons ribosomaux. Cependant, pour qu'ils aient une signification physiologique, les R-loops doivent exister dans une souche de génotype sauvage. L'analyse du plasmide pNO1302 extrait d'une souche sauvage montre la présence de conformères, ce qui n'est pas le cas du contrôle pEM501, où la transcription de l'opéron est absente (E. Massé, mémoire de maîtrise). De plus, la présence du pNO1302 dans les cellules

sauvages affecte significativement leur croissance. Néanmoins, la surproduction de la RNase H permet de corriger ce défaut. Il est probable que la RNase H et la topoisomérase I, présentes à des niveaux respectifs normaux, soient titrées par la transcription de l'opéron *rrnB* du pNO1302. Cette situation peut effectivement favoriser l'accumulation de R-loops sur le chromosome.

La formation de R-loops durant l'élongation de la transcription semble donc une conséquence importante de l'absence de la topoisomérase I chez *E. coli*. Cependant, les études reliées au rôle de la topoisomérase I durant l'élongation de la transcription ont généralement été menées à l'aide de mutants *topA* portant plusieurs mutations compensatoires. Puisque ces mutations peuvent cacher certains phénotypes, il est probable que les études précédentes ne pouvaient déceler le vrai rôle de la topoisomérase I. Nous avons donc utilisé une série de mutants *topA*, avec ou sans mutations compensatoires, qui présentaient des taux de croissance variés et nous avons comparé la distribution de leurs topoisomères plasmidiques. De cette façon, il est possible d'établir une corrélation entre le surenroulement (global ou local) et la capacité à croître des mutants.

Les résultats présentés dans Massé et Drolet (1999a) suggèrent principalement que l'augmentation du surenroulement global de l'ADN observée chez certains mutants *topA* n'est pas reliée au phénotype d'inhibition complète de la croissance. Les données convergent plutôt sur la nécessité de relaxer les supertours négatifs produits durant l'élongation de la transcription. L'accumulation de ces supertours, décelable par les topoisomères hypernégatifs, est en parfaite corrélation avec l'inhibition de croissance observée chez les mutants *topA*. Il est intéressant de remarquer que cette corrélation inclut les topoisomères hypernégatifs dépendants de la transcription, peu importe si le produit du gène s'attache à la membrane ou non. Selon ces informations, il est fortement vraisemblable que chez les mutants *topA*, les mutations compensatoires dans les allèles *gyrA* et *gyrB* surviennent principalement dans le but de diminuer l'activité de la gyrase durant l'élongation de la transcription. La relaxation du surenroulement global de l'ADN, liée à ces mutations compensatoires, ne serait qu'une conséquence de moindre importance. Un argument

important qui supporte cette hypothèse est que la surproduction de la RNase H, bénéfique pour la croissance des mutants *topA* (Drolet *et al.*, 1995), élimine les topoisomères hypernégatifs dépendants de la transcription, mais ne corrige pas le surenroulement global élevé.

Les résultats présentés dans Massé et Drolet (1999b) démontrent que la formation de R-loops *in vitro* sur le plasmide pBR322 ne semble pas affectée par le surenroulement global de l'ADN. En effet, aucun hybride ne se forme, lors de la transcription *in vitro* par la polymérase ARN de *E. coli*, sur cette matrice portant une densité de surenroulement négative plus élevée que celle d'un mutant *topA*. Dans ce système, le processus de formation des R-loops requiert obligatoirement l'activité de surenroulement de la gyrase. Dans les autres systèmes d'étude de la formation d'hybrides ARN-ADN tels ColE1, *oriC*, l'origine de réplication des mitochondries ou dans la commutativité des immunoglobulines, l'appariement de l'ARN naissant était relié à la séquence nucléotidique. Cependant les hybrides étudiés dans Massé et Drolet (1999b) sont probablement reliés au « twin-supercoiled-domain », via l'action des topoisomérases. Dans ce contexte, le mouvement de la polymérase ARN créer, derrière elle, une région où le surenroulement négatif généré permet la séparation des brins ADN. L'inertie conférée par la masse de l'ARN naissant participe également à augmenter la densité de surenroulement. Ainsi, l'ouverture des brins ADN constitue une situation dans laquelle l'ARN pourra s'apparier au brin ADN homologue. Il s'agit donc d'un système de formation de R-loops assez général, c'est-à-dire indépendant de la séquence nucléotidique ou de la densité de surenroulement global de l'ADN, bien que ces deux derniers facteurs peuvent aussi contribuer à faciliter l'ouverture de la double hélice et éventuellement la formation d'hybrides.

Ce concept d'hybridation de l'ARN naissant au brin ADN non apparié est soutenu par des données de transcription *in vitro* (Daube and von Hippel, 1994). Dans ces expériences, on observait le réappariement fréquent de l'ARN naissant avec une bulle de transcription artificielle et permanente qui avait servit à initier la transcription. Dans notre système, la région 5' de l'ARN naissant s'apparie avec son brin ADN homologue, de cette manière, la polymérase ARN se trouvera empêchée de

toute rotation. Selon le modèle du « twin-supercoiled-domain » cette situation générera encore plus de supertours négatifs (Liu and Wang, 1987; section 6.2.2). Une fois initié, l'hybride ARN-ADN pourra s'allonger grâce à l'activité de la gyrase qui premièrement, enlève les supertours positifs devant la polymérase et deuxièmement, introduira des supertours négatifs dans la matrice relaxée par l'hybride. Ces deux actions indépendantes permettront l'accumulation importante de surenroulement négatif dans la matrice tout en favorisant l'allongement du R-loop. Afin de s'allonger, l'ARN de l'hybride doit forcément tourner pour former une double hélice stable avec le brin ADN homologue. La fonction séparatrice de l'ARN à la matrice peut être rompue lorsque l'ARN rejoint la bulle de transcription. Il est possible que cette situation soit la même dans le cas d'autres systèmes qui utilisent la polymérase ARN de *E. coli* (ColE1, *oriC*).

Dans un mutant *topA*, la dégradation des R-loops par la RNase H, à une concentration cellulaire normale, ne peut contrecarrer l'action stimulante de la gyrase sur la formation des hybrides. C'est pourquoi elle doit être surproduite afin de rapidement éliminer les hybrides nouvellement initiés avant que la gyrase ne puisse les allonger. Cette situation peut même expliquer pourquoi on observe, en présence de spectinomycine, une distribution bimodale des topoisomères. Lorsque le R-loop est initié, et que la RNase H ne le dégrade pas immédiatement, l'allongement de l'hybride se fait très rapidement à l'aide de la gyrase, ce qui produit de l'ADN hypernégatif. Si le R-loop est dégradé au moment de l'initiation, le surenroulement de la matrice reste le même. Ainsi, on obtiendra un résultat « tout ou rien » qui créera deux populations distinctes. Dans le second cas, en présence de traduction, les ribosomes permettront la génération de supertours positifs éventuellement éliminé par la gyrase. Ce contexte, plus graduel, donnera une distribution hétérogène des topoisomères.

En absence de la RNase H et de la topoisomérase I, la formation de R-loops sera favorisée par l'activité de la gyrase, même si son activité est significativement diminuée. En effet, le double mutant *topA-rnhA* n'est pas viable (Drolet *et al.*, 1995), et ce, même dans une souche comme la DM800 qui possède une gyrase moins active,

en plus d'autres mutations compensatoires, et dans laquelle le surenroulement global est plus bas que dans une souche sauvage (Massé and Drolet, 1999c).

Certaines conditions de croissance peuvent modifier le surenroulement de l'ADN bactérien, ce qui permet à la cellule de moduler l'expression de promoteurs spécifiques afin de s'adapter aux nouvelles conditions. Ces promoteurs vont permettre l'expression de gènes qui rendent la cellule mieux adaptée aux diverses conditions environnementales dont, par exemple, les changements osmotiques (McClellan *et al.*, 1990; Higgins *et al.*, 1989), un passage d'incubation aérobie à anaérobie (Dorman *et al.*, 1988; Hsieh *et al.*, 1991) et la déplétion de sources nutritives (McClellan *et al.*, 1990). La topoisomérase I, en particulier, semble impliquée dans la réponse à des modifications des conditions de croissance comme la pression osmotique et la température (Graeme-Cook *et al.*, 1989; Ni Bhriain and Dorman, 1993), les « shift-ups » nutritionnels (Drolet *et al.*,1995) et la concentration en oxygène (Yamamoto and Droffner, 1985).

Le gène codant pour la topoisomérase I comporte cinq promoteurs connus, P1, P2, P3, P4 et PX1. Les quatre premiers promoteurs montrent une expression accrue lorsque le surenroulement augmente (Lesley *et al.*, 1990). Trois de ces promoteurs, P2, P3 et P4 présentent un consensus modéré à σ^{70}, alors que le promoteur PX1 est spécifique à la phase stationnaire (voir plus loin) et, finalement, le promoteur P1 requiert le facteur « heat-shock » σ^{32} (Lesley *et al.*, 1990). Cependant, malgré l'expression plus élevée de P1 durant un « heat-shock », il a été déterminé que la synthèse de la topoisomérase I reste constante, probablement due à la baisse d'expression simultanée du promoteur P4. La topoisomérase I n'est donc pas considérée comme une protéine « heat-shock », puisque sa concentration n'augmente pas durant un choc thermique.

La délétion du promoteur P1 du gène *topA* se traduit par une sensibilité accrue des bactéries aux température élevées (Qi *et al.*, 1996). En effet, lorsqu'elles sont incubées à 52°C, ces cellules sont 10 à 100 fois moins résistantes que les cellules sauvages. De plus, l'expression des protéines « heat-shock » DnaK et GroEL,

impliquées dans la tolérance aux températures élevées, est fortement diminuée dans les souches sans promoteur P1.

Une baisse drastique de la température d'incubation de 37°C à 10°C provoque, chez *E. coli*, un arrêt de la synthèse protéique et de la croissance cellulaire pouvant durer de 4 à 5 heures (« cold-shock »). Pendant ce temps, seulement quelques protéines continuent à être synthétisées. Il n'est pas nécessaire cependant que la température d'incubation passe de 37°C à 10°C pour observer une réponse « cold-shock ». En effet, une baisse de 13°C et plus provoquera une expression accrue des protéines « cold-shock » et réprimera les protéines « heat-shock » (Jones *et al.*, 1992a). Lors d'une réponse « cold-shock », la cellule augmente de 2 à 10 fois la synthèse des protéines NusA, RecA, H-NS, GyrA, et des facteurs d'initiation de la traduction 2β et 2α, alors que CS7.4 augmente de 10 à 100 fois (Goldstein *et al.*, 1990; Jones *et al.*, 1987). La protéine CS7.4 a été suggérée comme étant un régulateur positif de la transcription des gènes codant pour les protéines « cold-shock » (La Teana *et al.*, 1991). Par exemple, le promoteur du gène *gyrA* porte des séquences de régulations nommées « Y-box » qui, en présence de CS7.4, sont responsables de l'augmentation de la synthèse de GyrA (Jones *et al.*, 1992b). Les mêmes travaux ont aussi montré que la synthèse de la sous-unité GyrB augmente en état de « cold-shock ».

Le gène *rbfA* fut découvert comme étant un suppresseur multicopie d'une mutation dominante sensible au froid (« cold-sensitive ») située dans l'ARNr 16S (Dammel and Noller, 1995). Les auteurs suggéraient que la protéine RbfA devait aider à la maturation des ribosomes ou être un facteur d'initiation de la traduction. Les travaux de Jones et Inouye (1996) ont montré que cette protéine jouait un rôle dans la réponse « cold-shock ». Après une baisse importante de la température d'incubation, il est possible d'observer un arrêt momentané de la traduction. Dans ces conditions, le nombre de polysomes (ARNm portant plusieurs ribosomes) diminue alors que la quantité de sous-unités ribosomales 30S et 50S libres ainsi que du monosome 70S augmente. Le nombre accru de ribosomes incapables d'initier la traduction permet l'induction de la réponse « cold-shock ». À ce moment, la synthèse

de la majorité des protéines s'arrête, contrairement aux protéines impliquées dans la réponse « cold-shock » qui augmente. Il est probable que les ARNm codant pour ces protéines possèdent une affinité supérieure pour les ribosomes. Selon ces résultats, la protéine RbfA est nécessaire afin de permettre la formation du complexe d'initiation de la traduction des ARNm qui ne sont pas impliqués dans la réponse « cold-shock ». La protéine RbfA, de concert avec d'autres protéines « cold-shock », s'associeraient aux ribosomes non fonctionnels, afin de les rendre résistants au froid et capables de traduire.

L'étude de mutants *topA* nous a permit d'observer que la formation de structures hybrides ARN-ADN semblait favorisée lors d'une incubation à basse température, et ce, dans plusieurs types de mutants *topA* avec ou sans mutations températures sensibles. Ces résultats ne sont vraisemblablement pas reliés à une réponse « cold-shock » puisque nous observons l'accumulation d'ADN hypernégativement surenroulé malgré l'inhibition de la synthèse protéique conférée par l'ajout de spectinomycine. Des travaux récents sur la réponse « cold-shock » ont cependant montré que, malgré la présence d'antibiotiques bloquant la traduction, certaines des protéines impliquées dans l'adaptation au froid sont exprimées (Etchegaray and Inouye, 1999). Néanmoins, les données concernant la vitesse de formation de topoisomères hypernégatifs montrent qu'ils surviennent plus rapidement (10 min., Massé and Drolet, 1999c) que le temps requis pour amorcer la réponse « cold-shock ». La formation de topoisomères hypernégativement surenroulés est donc indépendante de la réponse de la cellule au froid.

La formation de topoisomères hypernégatifs à basse température peut s'expliquer à l'aide du modèle de formation de R-loops (voir section 6.2.7), de concert avec les résultats publiés par Daube et von Hippel (1994). En effet, ces derniers ont démontré que la réduction de la vitesse du complexe de transcription favorisait l'appariement de l'ARN nouvellement transcrit à son brin ADN homologue. Ces résultats sont en parfait accord avec ceux expliquant la formation de l'hybride initiateur de la réplication de l'origine ColE1, qui suggèrent aussi qu'une diminution de la vitesse de transcription favorise l'appariement de l'ARN naissant à

son brin ADN homologue (Masakuta and Tomizawa, 1990). Comme la température d'incubation, dans nos expériences, est largement sous le seuil optimal, il est très probable que ceci diminue la vitesse d'élongation de la polymérase ARN et, incidemment, favorise l'appariement du transcrit à la matrice. De plus, comme la traduction est découplée de la transcription lors d'une baisse de température, l'ARN devient alors libre de s'hybrider avec la matrice ADN à l'arrière de la polymérase. Nous proposons que la topoisomérase I puisse agir à ce moment afin d'éviter la formation de R-loops inhibiteurs de la croissance.

Une adaptation importante que les bactéries doivent aussi subir est l'entrée en phase stationnaire. Un promoteur régulé par le signal de phase stationnaire σ^S a été découvert récemment chez le gène *topA* de *E. coli* (Qi *et al.*, 1997). Lors de l'entrée en phase stationnaire de la bactérie *E. coli*, la production de protéines diminue considérablement. On observe cependant, durant cette phase, qu'il y a entre 50 et 100 protéines dont la synthèse est plus ou moins induite (Kolter *et al.*, 1993). La production de plusieurs de ces protéines est sous le contrôle d'un promoteur spécifique à RpoS (σ^S; Hengge-Aronis, 1996b). Dernièrement, il a été observé que les promoteurs répondant à σ^S, de même que le gène *rpoS*, sont impliqués dans la réponse à différents stress incluant l'osmolarité élevé, le pH acide, et les « heat-shock » (Marshall *et al.*, 1998). Dorénavant, le facteur σ^S n'est plus considéré comme un simple signal d'entrée en phase stationnaire, mais plutôt comme un régulateur global de réponse aux stress (Hengge-Aronis, 1996ab). Lors de l'entrée en phase stationnaire de la cellule, l'activité des promoteurs P2 et P4 de *topA*, reconnus par σ^{70}, diminuent fortement alors que le PX1 devient progressivement le principal site d'initiation de la transcription du gène. Les résultats de cette étude suggèrent que le niveau intracellulaire de topoisomérase I semble stable tout au long du cycle de croissance bactérien allant de la phase logarithmique jusqu'à la phase stationnaire.

Nos résultats ont démontré que, chez la souche DM800 *gyrB+* en phase logarithmique, la surproduction de la RNase H améliore grandement la croissance à 37°C, mais cependant, provoque l'entrée prématurée des cellules en phase stationnaire (Massé and Drolet, 1999c). Ce dernier phénotype peut cependant être corrigé par la

surproduction de la topoisomérase III (Broccoli *et al.*, 2000). Il est possible que dans une souche où la topoisomérase I est absente et la RNase H est surproduite, certains ARNm nécessaires pour l'entrée en phase stationnaire normale soient dégradés par la RNase H. La topoisomérase III surproduite éliminerait l'effet négatif de la RNase H en empêchant la dégradation de ces ARNm.

Les résultats des expériences sur les rôles de la topoisomérase I suggèrent que cette enzyme est probablement d'avantage un facteur de transcription qu'un régulateur de surenroulement global de l'ADN. En accord avec cette proposition il fut récemment démontré que la topoisomérase I de *E. coli* et la sous-unité β' de la polymérase ARN étaient copurifiées avec la transposase du transposon Tn5. De plus, il fut aussi noté que dans un mutant *topA*, la copurification de la polymérase avec la transposase diminue de 20 à 30 fois (Yigit and Reznikoff, 1999). Ce résultat suggère une interaction physique entre la topoisomérase I et la polymérase ARN chez *E. coli*. Chez d'autres organismes, plusieurs évidences montrent que les topoisomérases jouent un rôle important durant la transcription. Chez les drosophiles par exemple, la topoisomérase I est localisée aux sites actifs de transcription (Fleischmann *et al.*, 1984; Gilmour *et al.*, 1986). Chez les humains (Zhang and Liu, 1988) et l'organisme Tetrahymena (Gocke *et al.*, 1983), l'implication de la topoisomérase I a aussi été démontrée durant l'élongation de la transcription des opérons ribosomaux.

Dans un système *in vitro* humain, il fut démontré que la topoisomérase I était un activateur de la transcription (Merino *et al.*, 1993). L'activité de relaxation de la topoisomérase I n'est pas nécessaire afin d'activer la transcription. Il a été démontré *in vivo* que la présence d'anticorps spécifiques à la topoisomérase I provoque un blocage de l'élongation de la transcription (Egyhazi and Durban, 1987). Des résultats de coimmunoprécipitation ont montré que l'enzyme interagit avec le complexe TFIID et les TBP (« TATA binding protein »; Merino *et al.*, 1993). Ces données ont permis de suggérer que la topoisomérase I peut agir tant au niveau de l'initiation que de l'élongation de la transcription. Récemment, il fut démontré que la topoisomérase I favorisait la formation du complexe TFIID-TFIIA au promoteur, ce qui accentue

l'activation de la transcription (Shykind *et al.*, 1997). Ce dernier résultat supporte les énoncés décrits plus haut.

9   Conclusion et perpectives

Dans les travaux présentés ici, nous nous sommes penchés principalement sur le mécanisme de formation des R-loops et, plus spécifiquement, sur le rôle du surenroulement de l'ADN et des topoisomérases sur leur formation. Il sera maintenant intéressant d'étudier les effets des R-loops sur la physiologie des cellules. Ces études pourraient révéler des mécanismes par lesquels les topoisomérases et la topologie de l'ADN affectent les différentes fonctions de l'ADN. En effet, tel qu'illustré à la figure 3, les R-loops peuvent avoir plusieurs effets sur les fonctions de l'ADN. Ces conséquences se montrent souvent néfastes pour la cellule et, pour l'instant, nous pouvons suggérer que la formation d'hybrides ARN-ADN sur les opérons *rrn* n'est probablement pas l'unique cause de l'absence de croissance des mutants *topA*. De récents résultats provenant de notre laboratoire ont démontré que la transcription des opérons *rrn* semblait bloquée chez les mutants *topA gyrB*(Ts) incubés à 27°C et moins. Il est intéressant de remarquer que la surproduction de la RNase H corrige cet arrêt de transcription qui survient chez les mutants *topA*, sans toutefois corriger leur croissance à 21°C (Hraiky *et al.*, 2000). Nous avons vu que la traduction se trouve arrêtée lors d'une incubation à basse température. Dans ces conditions, la structure R-loop peut vraisemblablement se former dans des gènes peu exprimés et l'élimination de ces ARN par la RNase H sera évidemment plus néfaste qu'au niveau des opérons *rrn* très transcrits. Les mutants *topA* démontrent des difficultés d'adaptation aux changements de conditions de croissance, telle la baisse de température, il est donc vraisemblable que la topoisomérase I soit requise à ce moment afin de prévenir la formation d'hybrides durant la transcription.

La formation de R-loops avec un ARN normalement traduit peut expliquer que la surproduction de la RNase H, chez les mutants *topA* incubés à basse température (21°C), provoque une diminution drastique de la croissance. Puisqu'un découplage entre les ribosomes et la polymérase ARN est observé dans ces conditions de croissance, il est possible que la RNase H élimine les ARNm appariés à l'ADN. Afin de déterminer la validité de cette hypothèse, l'extraction des protéines et leur

Région ADN simple-brin
-Induction de la réponse SOS
-Recombinaison homologue

Barrière physique
-Bloquage de la transcription
-Bloquage de la réplication

Initiation de la réplication
-La structure R-loop est utilisée
comme origine de réplication
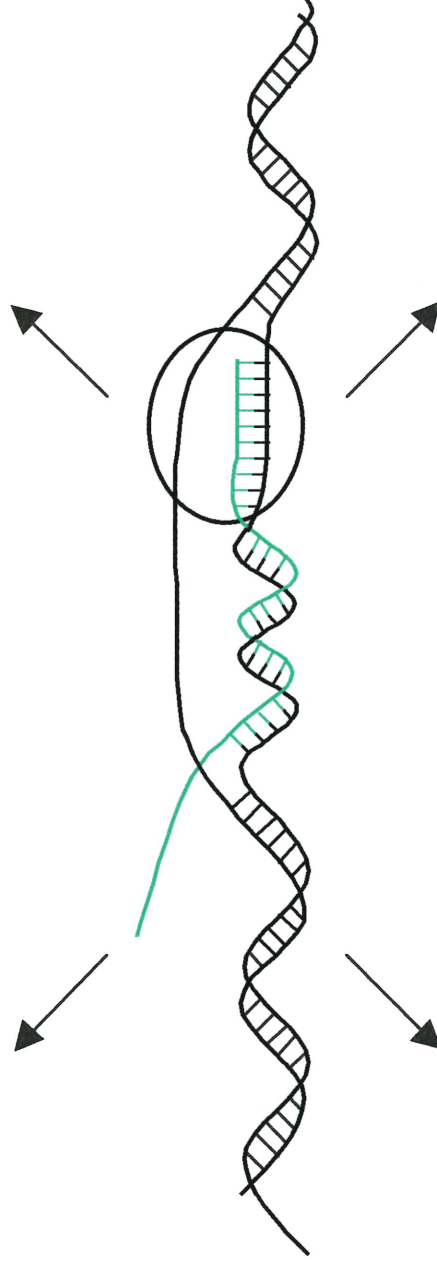
Inhibition de la synthèse protéique
-ARN non disponible pour la traduction
-Dégradation de l'ARN par la RNase H

Figure 3. Effets possibles des R-loops sur les fonctions de l'ADN.

analyse sur gel à haute résolution (deux dimensions) permettra de comparer leur expression individuelle, lorsque la RNase H est surproduite ou non. Ainsi, il sera possible de déduire quels sont les ARNm touchés par la dégradation due à la RNase H dans les mutants *topA*.

Il est vraisemblable qu'un hybride ARN-ADN puisse aussi empêcher la progression d'une fourche de réplication et, conséquemment, produire une brisure double-brin dans l'ADN lors de l'arrêt de la fourche. La réparation de ces lésions, chez *E. coli*, est réalisée à l'aide des protéines RecA et RecBCD. Donc, à l'aide d'un tel système *in vivo*, on peut étudier les mécanismes de formation et de réparation des brisures double-brin dans l'ADN, et ainsi améliorer notre compréhension du rôle des différentes composantes qui sont impliquées.

La structure R-loop peut, par exemple, amener plusieurs avantages particuliers pour la cellule bactérienne. Le premier de ces avantages, la réplication stable et constitutive, permet peut-être à la cellule de s'adapter rapidement aux différentes conditions de croissance (réplication de l'ADN plus rapide, dosage génique des gènes localisés dans les régions répliquées). La cellule portant plusieurs opérons ribosomaux peut vraisemblablement croître plus rapidement. Aussi, il est possible que l'amplification des opérons ribosomaux dans les mutants *topA* puisse simplement représenter une adaptation afin de diminuer le taux de transcription individuel des sept opérons ribosomaux réduisant alors la quantité de R-loops formés. De plus, si un hybride se forme tout de même sur l'un d'eux, empêchant son expression, le reste des opérons pourra compenser ce blocage.

Selon les résultats de notre laboratoire, la protéine RecA semble favoriser la stabilisation de l'ouverture des brins ADN une fois l'hybride ARN-ADN formé. Il est aussi possible que malgré l'élimination de l'hybride par la RNase H, la protéine RecA puisse laisser, dans l'ADN, l'ouverture pratiquée par le R-loop. Ceci peut permettre d'augmenter les chances de recombinaison, par exemple entre les opérons *rrn*.

Il a été démontré, chez les procaryotes, que la recombinaison entre différents opérons *rrn* résultait en une duplication en tandem des opérons ainsi que des gènes

voisins (mérodiploïdie; Hill *et al.*, 1977). Ce type de duplication survient spontanément à une fréquence relativement faible chez les cellules sauvages (de 0.01% à 0.3%; Anderson and Roth, 1981). Nos données préliminaires ont démontré que chez les mutants *topA*, la fréquence de duplication en tandem des opérons ribosomaux pouvait augmenter significativement (de 20 à 50 fois; É. Massé et M. Drolet, résultats non publiés). Il est possible que la présence de R-loops dans les opérons *rrn* puisse favoriser les évènements de recombinaison. Il reste cependant à déterminer si l'augmentation du taux de duplication observée chez les mutants *topA* est reliée directement à la présence de R-loops ou s'il s'agit d'une conséquence indirecte.

Malgré les évidences provenant des systèmes eucaryotes, l'interaction entre la topoisomérase I et la polymérase ARN chez *E. coli* n'a pas été démontrée de manière directe. Les travaux de Yigit et Reznikoff (1999) ont été les premiers à suggérer une interaction possible de ces deux enzymes. Il serait intéressant de procéder à des analyses plus directes, à l'aide de techniques comme la coimmunoprécipitation, afin de démontrer l'interaction entre ces deux protéines, ce qui renforcerait l'hypothèse que le rôle principal de la topoisomérase I est au niveau de la transcription.

L'étude des mutants *topA* a donc permis de développer des systèmes d'études afin de mieux comprendre la transcription, la réplication et la recombinaison chez les bactéries. De plus, la collaboration étroite de la traduction avec la transcription, afin d'éviter la formation de R-loops, a pu être mis en évidence grâce a ce système. Les prochaines étapes pourront probablement mettre encore plus en lumière ces fonctions du chromosome.

10 Bibliographie

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