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Anchoring of DNA to the Bacterial Cytoplasmic Membrane through Cotranscriptional Synthesis of Polypeptides Encoding Membrane Proteins or Proteins for Export: a Mechanism of Plasmid Hypernegative Supercoiling in Mutants Deficient in DNA Topoisomerase I

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A homologous set of plasmids expressing *tet*, *lacY*, and *melB*, genes encoding integral cytoplasmic membrane proteins, and *tolC* and *ampC*, genes encoding proteins for export through the cytoplasmic membrane, was constructed for studying the effects of transcription and translation of such genes on the hypernegative supercoiling of plasmids in *Escherichia coli* cells deficient in DNA topoisomerase I. The results support the view that intracellular bacterial DNA is anchored to the cytoplasmic membrane at many points through cotranscriptional synthesis of membrane proteins or proteins designated for export across the cytoplasmic membrane; in the latter case, the presence of the signal peptide appears to be unnecessary for cotranscriptional membrane association.

The phenomenon of hypernegative supercoiling of plasmids in *Escherichia coli topA* mutants lacking DNA topoisomerase I was first reported by Pruss (37). The widely used cloning vector pBR322 isolated from *topA* null mutants was found to exhibit an extremely heterogenous distribution in its linking number, with a large fraction of the topoisomers more than twice as negatively supercoiled as the same plasmid isolated from isogenic $topA^+$ strains. This *topA*-dependent hypernegative supercoiling is plasmid specific: topoisomers of pUC19, a shortened derivative of pBR322, exhibit only minor differences in their linking numbers when isolated from isogenic *topA* mutant and $topA^+$ strains. Dissection of the pair of plasmids pBR322 and pUC19 led Pruss and Drlica (38) to conclude that transcription of *tet*, the gene encoding the tetracycline resistance marker in pBR322, is necessary for the hypernegative supercoiling of the plasmid in the absence of DNA topoisomerase I; various deletions within the *tet* region also show that a functional product of the gene is not necessary for the phenomenon.

The findings of Pruss and Drlica (38) provided a key experimental link between transcription and DNA supercoiling. Theoretical considerations on a plausible relation between transcription and DNA mechanics, however, can be traced two decades back. The idea that transcription might require a swivel in the DNA template to facilitate the rotation of the DNA relative to the RNA polymerase was first discussed in the 1960s (29; see also reference 13). With the discovery of *E. coli* DNA topoisomerase I in 1971, then known as the ω protein (52), the possibility that this enzyme is involved in transcription was raised in this connection (53). More recently, it was postulated that a highly negatively supercoiled loop might form in the DNA template when the RNA polymerase is in contact with a template-bound regulatory protein (54).

In 1987, Liu and Wang (25) proposed a twin-supercoiled-domain model of transcriptional supercoiling to account for all known experimental findings, including both the hypernegative supercoiling phenomenon described above and the observation of Lockshon and Morris (26) that inhibition of DNA gyrase in *E. coli* leads to the formation of highly positively supercoiled pBR322. The model postulates that under certain conditions, to be described below, a transcribing RNA polymerase and its nascent transcript might be prevented from circling around the DNA template, the consequence of which is that positive supercoils would be generated in the template ahead of the translocating polymerase and negative supercoils would be generated behind it. The model predicts that the accumulation of supercoils in the DNA template would be countered by their removal by enzymatic processes as well as by diffusional pathways; preferential binding of molecules to negatively or positively supercoiled domains, or supercoiling-driven structural transitions in the DNA double helix, may also influence the state of supercoiling of intracellular DNA. In bacteria, DNA topoisomerase I would specifically remove negative supercoils behind the RNA polymerase and DNA gyrase would specifically remove the positive supercoils ahead of the polymerase; in eukaryotes, either topoisomerase I or II can remove negative or positive supercoils. Alternatively, two oppositely supercoiled domains could annihilate each other by rotational diffusion of the DNA segment connecting the two domains; the effectiveness of such a diffusional pathway is dependent on the kind of macromolecules associated with the connecting segment and on whether the segment is anchored to a cellular structure.

Liu and Wang (25) described two types of mechanisms that could hinder the circling of the polymerase around its DNA template and thus effect template supercoiling. One involves the anchoring of the polymerase or macromolecules associated with it on the template (34, 54) or the anchoring of both the transcriptional ensemble and a point or points on the DNA template to a large cellular structure (25, 55). The other can be viewed as a special case of the diffusional

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pathways discussed above for the annihilation of oppositely supercoiled domains: a very long nascent transcript with its associated protein molecules, for example, might encounter a large frictional force against its circling around the DNA. When the transcriptional ensemble itself is the diffusional barrier, then a minimum of two polymerase molecules moving in opposite directions, or moving asynchronously in the same direction, could divide a circular DNA into two oppositely supercoiled domains (25).

One of the plausible modes of anchoring involves the synthesis of a message encoding a membrane protein in bacteria. Liu and Wang (25) pointed out that simultaneous transcription and translation in bacteria and cotranslational insertion of the nascent polypeptide into the membrane, events which hereupon will be referred to as cotranscriptional membrane association, would prevent the nascent mRNA and the polymerase attached to it from rotating around the DNA. Mutational analysis of the *tet* region of pBR322 led Lodge et al. (27) to conclude that hypernegative supercoiling of the plasmid in *topA* mutant cells requires transcription and translation of the beginning part of the *tet* gene; on the other hand, divergent transcription of the *bla* gene encoding ampicillin resistance, from either the P_{bla} or $P_{anti\text{-}tet}$ promoter, is unnecessary. Lodge et al. (27) therefore argued that cotranscriptional membrane association of the amino terminal of the nascent Tet polypeptide would serve as one barrier to the diffusional dissipation of supercoils in the circular DNA and that there might be a second such barrier in the DNA segment containing the origin of replication, due to either a high rate of transcription within this segment or the binding of the origin to the cytoplasmic membrane.

The key feature of the cotranscriptional membrane association interpretation, namely, membrane anchoring of the transcription-translation complex mediated by the nascent Tet protein, lacked direct experimental evidence. The beginning part of the coding region of the Tet mRNA, for example, could invade the DNA template and thus anchor the entire message, which is one of the other anchoring possibilities suggested by Liu and Wang (25). Several mechanisms are plausible for an RNA to invade a DNA directly; examples are triplex formation between RNA and duplex DNA, duplex formation between RNA and DNA in conjunction with DNA H-form formation (41), and R-loop formation. In the classical study of the initiation of ColE1 DNA replication by a primer RNA, it is well established that the invasion of a duplex DNA by a transcript is critically dependent on the nucleotide sequence and the structure of the folded nascent transcript (31).

The amino acid sequence of the Tet protein suggests that it is a member of a class of *E. coli* integral cytoplasmic membrane proteins which characteristically possess 12 transmembrane segments (5, 6, 11, 36, 58, 59). Lodge et al. (27) suggested that the amino-terminal 98-amino-acid stretch of the Tet protein, which is predicted to form the first three transmembrane domains (15), was required for cotranscriptional membrane association. They found that in-frame fusion of this stretch to the coding sequences of *lacZ* was sufficient to effect hypernegative supercoiling but that the same in-frame fusion of the first 34 codons of *tet* to *lacZ* was insufficient. It was reported earlier by Pruss and Drlica (38), however, that in a pBR322 deletion derivative, pEV-B, the expression of a 122-amino-acid polypeptide beginning with the amino-terminal 34 amino acids of the Tet protein and ending with 88 amino acids encoded by an out-of-frame *tet* segment was sufficient to effect hypernegative supercoiling. Thus, if membrane anchoring of the Tet protein is responsible for hypernegative supercoiling, the first 34 amino acids of the protein corresponding to the first transmembrane domain would appear to be sufficient, though dependent in some unknown way on the context of the polypeptide following it.

If membrane anchoring is one major mechanism of hypernegative supercoiling, it seems likely that expression of proteins for export across the cytoplasmic membrane, as well as the expression of an integral membrane protein such as Tet, would lead to plasmid hypernegative supercoiling in *topA* mutants. It is well established, however, that the expression of the pBR322 *bla* gene encoding the periplasmic β-lactamase is neither necessary nor sufficient for hypernegative supercoiling of the plasmid (27, 37, 38).

In order to address these issues, we examined the effects of transcription of the genes *tet*, *lacY*, *melB*, *tolC*, and *ampC* and their derivatives, from an identical promoter, on the state of supercoiling of plasmids containing each of the different genes but which are otherwise identical. The results indicate that hypernegative supercoiling in *E. coli* mutants lacking DNA topoisomerase I is a fairly general phenomenon in cases involving the expression of either integral membrane proteins or proteins destined for export through the periplasmic membrane, provided that membrane association of these proteins is fast relative to the rate of cotranscriptional translation; the lack of an effect of the *bla* gene transcription on hypernegative supercoiling can be attributed to its posttranslational mode of export (22, 24).

MATERIALS AND METHODS

DNA manipulations. Standard molecular biology methods were employed throughout the course of these studies (for examples, see references 30 and 44). The sources and relevant characteristics of *E. coli* strains and plasmids used in the course of these studies are listed in Table 1. *E. coli* DH5α was used for routine propagation of plasmids. Oligonucleotides for DNA sequencing and amplification of DNA by the polymerase chain reaction (PCR) were synthesized with an Applied Biosystems model 391 DNA synthesizer or purchased from Oligos Etc. Enzymes for routine in vitro DNA manipulations were obtained from various sources; Amplitaq DNA polymerase (Cetus) was used for all PCRs, and phage T4 gene 32 protein (Pharmacia) was added to the PCR mixtures used in the amplification of the 3.1-kb *lacZ* open reading frame (ORF; see Table 2) under conditions recommended by Schwarz et al. (46).

pBR322Δtet was constructed in two steps; first, a *Not*I linker (5′ GCGGCCGC 3′) was cloned into the *Nru*I site of pBR322 to form pBR322NotI. pBR322NotI linearized with *Pvu*II was then used as a PCR substrate with a pBR322 *Pst*I clockwise primer (5′ ATTGTTGCCGGGAAGCTAGAGT AAGTAGTT 3′; New England Biolabs) corresponding to bases 3542 to 3571 and a counterclockwise primer (5′ TAT GCGGCCGCAGATCTCCATGGATCCTGCCTGACTGC GTTAGCAATTT 3′), of which the underlined sequence corresponds to nucleotides 79 to 58 in pBR322. The PCR product was digested with *Pst*I and *Not*I, and the 860-bp product was gel purified and ligated to the 2,641-bp *Pst*I-*Not*I fragment of pBR322NotI to yield the plasmid pBR322Δtet (see Fig. 1).

The unique *Bam*HI site (GGATCC) in pBR322Δtet abuts the *tet* gene ATG initiation codon. This site and the downstream *Not*I site served as the sites of insertion of appropriately tailored ORF cassettes of *lacY*, *melB*, *ampC*, *tolC*, and *lacZ*, all obtained by PCR using appropriate cloned genes or

TABLE 1. *E. coli* strains and plasmids used in these studies

Strain or plasmid	Genotype or other relevant characteristics	Source or reference
E. coli strains		
ASL56	*cysB*$^+$ *topA*$^+$ derivative of DM800	This work
ASL111	*zid-501*::Tn*10* derivative of DM800	This work
ASL115	*gyrB225*::Tn*10* derivative of BR83	This work
BR83	F$^-$ *argA rpsL lac*Δ*514 topA57*(Am) *supD43,74*	Richard E. Depew
CAG12184	*tolC210*::Tn*10*	M. Singer et al. (48)
CAG18499	*zid-501*::Tn*10*	M. Singer et al. (48)
DH5α	F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 supE44* λ$^-$ *thi-1 gyrA96 relA1*	Laboratory stock
DM800	Δ(*topA-cysB*)*204 acrA13 gyrB225*	Laboratory stock (9)
DW2	*melA*$^+$ Δ*melB* Δ*lacZY*	Thomas H. Wilson
HB101	*hsdR hsdM supE44 ara14 galK2 lacY1 proA2 rpsL20 xyl-5 recA13 mcrB*	Laboratory stock
KL16	Wild-type K-12 strain	Laboratory stock
NK5031	F$^-$ *sulII gyrA*(Nal^r) *lac*Δ*MM5265*	Nancy Kleckner
Plasmids		
pACYC184	Cloning vector	Laboratory stock (7)
pAX629	pACYC184 derivative containing the *E. coli tolC* (*mukA*) gene	Sota Hiraga (19)
pBR322	Cloning vector	Laboratory stock
pNU28	pBR322 derivative containing the *E. coli ampC* gene	Eveline Bartowsky (21)
pRS317	pBR322 derivative containing the *E. coli lacY* gene	Laboratory stock
pSELECT-1	Cloning vector	Promega Corp.

genomic DNA as templates (see Tables 1 and 2). In all cases the sequence of the downstream primer was chosen such that the PCR product contained a translation termination codon at the 3′ end but no known transcription termination signal sequences. Confirmation that the desired constructs had been obtained was done by restriction mapping and DNA sequencing of the 5′ or *Bam*HI side of the fusion junction regions. Functionality of the cloned genes was

TABLE 2. Derivatives of pBR322Δtet constructed

Plasmid	Gene inserted[a]	Sequence reference(s)	Primers used for gene amplification by PCR[b]
pBR322ΔtetlacY	*lacY*	EMBL file JO1636	1 24 Upstream: 5′-CGGGATCCATGTACTATTTAAAAAACACAAAC-3′ 1272 1254 Downstream: 5′-CCGCGGCCGCGCATCCGACATTGATTGC-3′
pBR322ΔtetmelB	*melB*	Yazyu et al. (59)	1 24 Upstream: 5′-CGAGATCTATGACTACAAAACTCAGTTATGGA-3′ 1432 1411 Downstream: 5′-CCGCGGCCGCTTTTCGTTAGCTATTGTCCACG-3′
pBR322ΔtetlacZ	*lacZ*	EMBL file JO1636	1 21 Upstream: 5′-CGGGATCCATGACCATGATTACGGATTCA-3′ 3074 3053 Downstream: 5′-CCGCGGCCGCTTATTTTTGACACCAGACCAAC-3′
pBR322ΔtetampC	*ampC*	Jaurin and Grundstrom (21)	1 21 Upstream: 5′-CGGGATCCATGTTCAAAACGACGCTCTGC-3′ 1211 1191 Downstream: 5′-CCGCGGCCGCTTCGGACCCGATGGAATTTTA-3′
pBR322ΔtetampCΔ	*ampC*Δ	Jaurin and Grundstrom (21)	61 81 Upstream: 5′-GCGGATCCATGGCCCCTCAACAAATCAACGAT-3′ 1211 1191 Downstream: 5′-CCGCGGCCGCTTCGGACCCGATGGAATTTTA-3′
pBR322ΔtettolC	*tolC*	Niki et al. (32); Hackett and Reeves (17)	1 22 Upstream: 5′-CGGGATCCATGAAGAAATTGCTCCCCATTC-3′ 1497 1480 Downstream: 5′-CCGCGGCCGCCCCGTCGTCGTCATCA-3′
pBR322ΔtettolCΔ	*tolC*Δ	Niki et al. (32); Hackett and Reeves (17)	67 88 Upstream: 5′-CGGGATCCATGGAGAACCTGATGCAAGTTTATC-3′ 1497 1480 Downstream: 5′-CCGCGGCCGCCCCGTCGTCGTCATCA-3′

[a] Genes inserted in between the *Bam*HI and *Not*I sites of pBR322Δtet. All PCR products except that of the *melB* gene were first digested with *Bam*HI and *Not*I, and the gel-purified DNA fragments were subsequently cloned in between the same sites in pBR322Δtet; the *melB* product, which contained an internal *Bam*HI site, was digested with *Bgl*II and *Not*I, and the gel-purified DNA were subsequently cloned in between the *Bam*HI and *Not*I sites of pBR322Δtet.

[b] The underlined sequence in each primer corresponds to that of the gene, and the numbers above each sequence refer to nucleotide positions derived from the sequence reference.

further confirmed by genetic complementation of *E. coli* strains containing well-characterized null mutations in the corresponding chromosomal alleles of the cloned genes, as described below. The expression of a functional lactose permease from pBR322ΔtetlacY was shown by complementation of the *lacY1* mutation of *E. coli* HB101; HB101 cells harboring pBR322ΔtetlacY formed red colonies on MacConkey lactose medium containing ampicillin (50 μg/ml). Similarly, the expression of a functional β-galactosidase from pBR322ΔtetlacZ was demonstrated by transforming *E. coli* NK5031 *lacZ* mutant cells to $lacZ^+$ by the white-to-blue color assay of colonies on Luria broth agar plates containing ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 40 μg/ml). The expression of a functional melibiose permease from pBR322ΔtetmelB was shown by complementation of the Δ*melB* mutation of *E. coli* DW2; at 30°C, DW2 cells harboring pBR322ΔtetmelB form pink colonies on MacConkey melibiose agar plates containing ampicillin (50 μg/ml) and form colonies on M9 minimal medium agar plates containing ampicillin (50 μg/ml) and melibiose (1%) as the sole carbon source. Because of the presence of the *bla* ampicillin resistance marker in the vector pBR322Δtet, demonstration of the functionality of the *ampC* cassette of pBR322ΔtetampC was achieved by subcloning the *Cla*I-*Eag*I *ampC* fragment from pBR322ΔtetampC into the cloning vector pACYC184 (7), replacing the *tet* region; the resulting plasmid, pACYC184ΔtetampC, was shown to confer both chloramphenicol and ampicillin resistance on recipient cells. Subcloning of the corresponding *Cla*I-*Eag*I fragment from pBR322ΔtetampCΔ into pACYC184 resulted in a construct (designated pACYC184ΔtetampCΔ) that conferred only chloramphenicol resistance on recipient cells, demonstrating that removal of the AmpC signal sequence functionally abolishes export. That pBR322ΔtettolC encodes a functional TolC protein was shown by complementation of the *tolC210* mutation of *E. coli* CAG12184: strain CAG12184 cells harboring pBR322ΔtettolC are able to grow on Luria broth plates containing ampicillin (100 μg/ml) and sodium deoxycholate (0.05%); the same cells harboring pBR322ΔtettolCΔ failed to grow on the same plates, indicating that removal of the TolC signal sequence functionally abolishes export.

Several additional plasmids were also constructed. pBR322ΔtetlacYΔC was derived from pBR322ΔtetlacY by the deletion of an internal *Afl*II fragment. Cutting the parent plasmid with *Afl*II and religating the resulting 4,130-bp fragment following the repair of its 3′ recessed ends with DNA polymerase I introduced an in-frame termination codon at the site of ligation. Thus, pBR322ΔtetlacYΔC expresses the amino-terminal half of lactose permease containing the first six transmembrane domains of the protein (3). To construct pBR322Bam, the *tet* region of pBR322 was first amplified by PCR using a pair of primers, 5′ CGGGATCCATGAAATCTAACAATGCGCTCATC 3′, the underlined portion of which corresponds to the first 24 coding nucleotides of *tet*, and 5′ GCTGGAGATGGCGGACGC 3′ (New England Biolabs), which corresponds to nucleotides 1390 to 1407 in pBR322. The 1,329-bp PCR product was digested with *Bam*HI and *Sty*I, and the resulting 295-bp *Bam*HI and 994-bp *Bam*HI-*Sty*I fragments were gel purified. Both fragments were then inserted in tandem between the *Bam*HI and *Sty*I sites in pBR322Δtet in a single ligation step. The resulting clone, pBR322Bam, is identical to pBR322 with the exception of five nucleotide substitutions in a 6-bp region immediately upstream of the *tet* ATG initiation codon (^{80}CCGTGT changed to ^{80}GGATCC). The expression of *tet* from this plasmid is therefore from the same transcription and translation signals as that of the other ORFs cloned into pBR322Δtet. pBR322Δ188-375 was constructed by digesting pBR322 with *Eco*RV and *Bam*HI, repairing the 3′ recessed end with DNA polymerase I, and religating the gel-purified 4,175-bp fragment. Deletion of the 187-bp *Eco*RV-to-*Bam*HI segment of pBR322 results in a plasmid in which P_{tet} directs the expression of a 122-amino-acid ORF beginning with the first 34 codons of the Tet protein and ending with 88 codons of the −1 reading frame of *tet* downstream of the *Bam*HI site; pBR322Δ188-375 is presumed to be identical to the pEV-B plasmid constructed by Pruss and Drlica (38).

pBR322Bam-1 is a derivative of pBR322Bam which was constructed by replacing the 1,140-bp *Nhe*I-*Sty*I fragment of pBR322Bam with the corresponding fragment derived from the *tet* region of pSELECT-1 (Promega). In pSELECT-1, the *Bam*HI, *Sal*I, and *Sph*I sites present in the wild-type pBR322 *tet* sequence have been removed by site-directed mutagenesis through the introduction of three nucleotide substitutions which do not alter the coding sequence. pBR322Bam-1 is therefore identical to pBR322Bam with the exception of the aforementioned three nucleotide substitutions.

Finally, a pool of mutant derivatives of pBR322Bam-1 in which nucleotide changes were introduced into the *tet* sequence corresponding to the first 34 codons was constructed. The nucleotide substitutions were designed to maximize changes in the DNA sequence of the region (and therefore the sequence of corresponding mRNA) without altering the sequence of the protein encoded. A degenerate 129-mer oligonucleotide, 5′ ATGGACGATATCTC(GT)(GAT)AG(TC)AA(ATC)CC(GAT)GGTAA(GAC)AC(GAT)GGCAT(GTC)AC(GAT)AG(ATC)CC(GA)AT(ATC)CCTACAGCGTCTAA(ATC)GT(ATC)AC(ACT)GT(ACT)CC(TC)AA(AT)AT(ATC)AC(AT)AT(TC)AA(GAT)GCGTTATTGCTCTTCATGGATCCTGCCTGACTGCG 3′, corresponding to the antisense *tet* strand of the region in pBR322Bam and within which the degenerate positions include all possible bases other than those present in the wild-type *tet* sequence (nucleotides in parentheses), was synthesized. The oligonucleotide was designed to introduce a total of 39 nucleotide changes in 29 of the 32 changeable codons. Nucleotide changes other than the degenerate ones are underlined in the 129-mer sequence; no changes in codons 18, 19, and 34 were made to conserve an *Sfc*I site and the unique *Eco*RV site for cloning purposes. The 129-mer degenerate oligonucleotide was amplified by PCR using a pair of primers: 5′ CGCAGTCAGGCAGGATCCATGA 3′, corresponding to nucleotides 68 to 89 of pBR322Bam, and 5′ GCTGTCGGAATGGACGATATCTC 3′, the underlined portion of which corresponds to nucleotides 205 to 185 in pBR322. The PCR product was digested with *Bam*HI and *Eco*RV, and the resulting 107-bp product was purified from an 8% polyacrylamide gel and ligated to the 4,255-bp *Bam*HI-*Eco*RV fragment of pBR322Bam-1. The ligation mixture was electroporated into *E. coli* DH5α, and tetracycline-resistant transformants were selected on Luria broth plates containing tetracycline (12.5 μg/ml). Two of the resulting clones, designated pBR322tet1 and pBR322tet2, were selected at random, and the nucleotide sequences of the mutated regions determined; each was found to contain 38 of the expected 39 nucleotide changes, and the two sequences differ only in the degenerate positions. The sequences specifying the third codon of Tet were found to be AGT in both mutants, instead of the expected AGC triplet specified by the 129-mer oligonucleotide; this was probably due to an error in the synthesis of the oligonucleotide.

Genetic manipulations. Transductions using phage P1*vir* were performed as described by Silhavy et al. (47). Transformation of *E. coli* with plasmid DNA was performed by standard chemical treatments (33, 44) or by electroporation as described by Zabarovsky and Winberg (60) with a Bio-Rad apparatus.

E. coli ASL115 was constructed by first transducing *E. coli* DM800 to tetracycline resistance by using a P1*vir* preparation from *E. coli* CAG18499, which contains a Tn*10* element closely linked to the *gyrB* locus. A P1*vir* preparation from one such transductant (designated ASL111) was then used to transduce *E. coli* BR83, and tetracycline-resistant transductants were selected at 30°C and subsequently screened for temperature sensitivity by replica plating at 30 and 42°C. The introduction of the *gyrB225* mutation of DM800 into BR83, which fails to synthesize a functional DNA topoisomerase I because of the presence of its *topA57*(Am) and *supD43*, *74* alleles (28), is expected to compensate for the temperature-sensitive growth phenotype of BR83 (9). Among the tetracycline-resistant transductants, 72% were found to be non-temperature sensitive for growth; *E. coli* ASL115 is one such transductant.

E. coli ASL56 is a $topA^+$ derivative of DM800 obtained by transducing the latter to cysteine prototrophy by using a P1*vir* preparation made from *E. coli* KL16. Transductants were screened for the presence of the *topA* gene by PCR using *topA*-specific amplimers. *E. coli* ASL56 was one such transductant.

Analysis of topoisomer distributions. Cells were grown in Luria broth containing 100 μg of ampicillin per ml to an apparent optical density of 0.4 to 0.6 at 600 nm (as measured in a Cary 118 spectrophotometer). Plasmid DNAs were isolated by the alkaline lysis method as described by Maniatis et al. (30). Two-dimensional gel electrophoresis was performed as described previously (35), using agarose gel slabs (20 by 20 cm) containing 1× TAE buffer (30) and chloroquine disphosphate at the concentrations indicated in the figure legends. Following electrophoresis, DNA was transferred from the gel to nylon membranes (Dupont Gene-Screen) as described by Reed and Mann (42) and hybridized with ^{32}P-labeled DNA probes prepared by random priming of an *Rsa*I digest of pBR322Δtet by using a kit supplied by Pharmacia. ^{32}P-labeled probe DNA that remained bound to the filters after high-stringency washing (30) was visualized by autoradiography.

RESULTS

Construction of *E. coli* strains and plasmids for the study of hypernegative supercoiling. Because of the strong dependence of plasmid hypernegative supercoiling on DNA topoisomerase I, we have constructed a temperature-dependent *topA* strain to facilitate the study of this phenomenon. ASL115 is a *gyrB225* derivative of strain BR83, which fails to synthesize a functional DNA topoisomerase I at 42°C because of the presence of the *topA57*(Am), *supD43*, and *supD74* alleles (28). Whereas the parent strain BR83 does not grow at 42°C because of the absence of DNA topoisomerase I, the presence of the *topA*-compensatory mutation *gyrB225* in ASL115 makes it viable at 42°C without a functional DNA topoisomerase I (see Materials and Methods for experimental details of the construction of this and other strains). A $topA^+$ derivative of strain DM800 (Δ*cysB* Δ*topA gyrB225*), ASL56, was also constructed to serve as an isogenic $topA^+$ control in experiments using strain DM800.

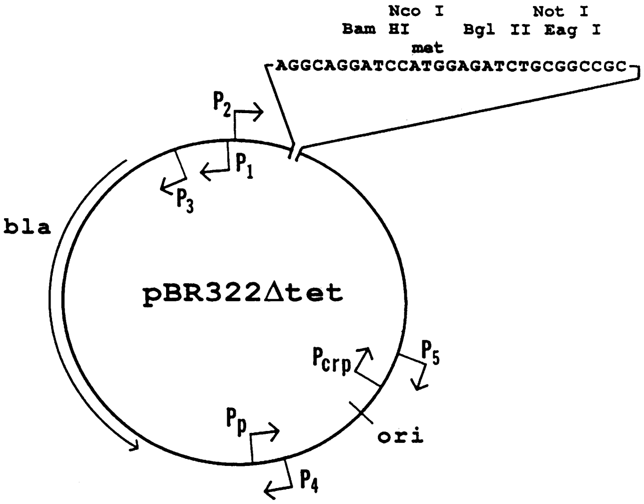
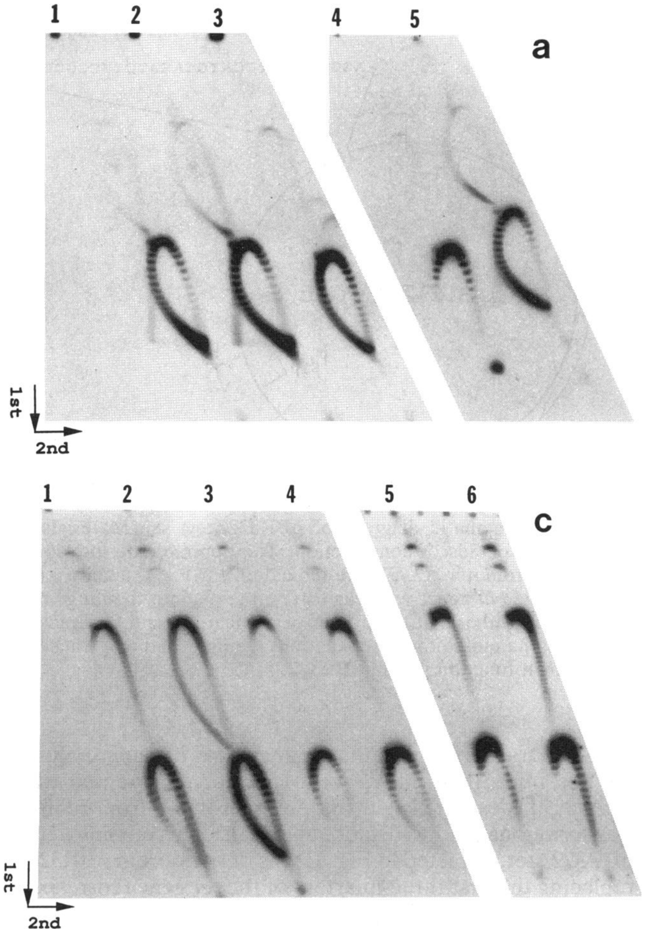


FIG. 1. Schematic diagram of pBR322Δtet; see Materials and Methods for details of construction. The approximate locations of all known promoters (2, 51) are indicated. P_1 ($P_{\text{anti-}tet}$) and P_3 (P_{bla}) read into the *bla* gene, whereas P_2 (P_{tet}) reads into the *tet* gene. P_p directs the synthesis of the primer of replication, and P_4 directs the synthesis of an inhibitor of replication. P_{crp} and P_5 direct transcripts of unknown function within pBR322.

To facilitate direct comparisons of the effects of plasmid-encoded transcription units on the state of supercoiling of the plasmids in *E. coli*, we constructed a vector which enables the expression of different proteins from identical transcriptional and translational signals. This cloning vector, pBR322Δtet, depicted in Fig. 1, was derived from pBR322 by replacing the first three-quarters of the *tet* gene (corresponding to nucleotides 80 to 972) with a polylinker sequence. The restriction sites included in the polylinker region were designed to facilitate simple subcloning of suitably tailored ORFs to be expressed. All known in vivo transcriptional regulatory elements of pBR322 (2, 51) are conserved in pBR322Δtet. The ORF cassettes cloned in between the *Bam*HI and *Not*I sites of pBR322Δtet are under transcriptional regulation of the P_{tet} promoter, and the resulting mRNAs are expected to contain the untranslated leader sequence of the normal *tet* mRNA (nucleotides 45 to 85 of pBR322). The inclusion of a *Bam*HI site immediately upstream of the *tet* ATG initiation codon, however, introduces five nucleotide substitutions, CCGTGT to GGATCC, which may affect the translational efficiency of the P_{tet}-directed transcripts (14, 16, 23). To show that these changes do not significantly alter the formation of highly negatively supercoiled plasmids in *E. coli topA* mutants, we have also constructed a control plasmid, pBR322Bam, which is identical to pBR322 with the exception of the five aforementioned nucleotide changes.

Hypernegative supercoiling of pBR322 *tet* mutants. Given the apparent discrepancies between the data of Pruss and Drlica (38) and that of Lodge et al. (27) regarding the minimal pBR322 *tet* sequence determinants required to effect hypernegative supercoiling of plasmids in *E. coli topA* mutant cells, we undertook a series of studies designed to further elucidate both the minimal *tet* sequence requirement and the mechanism by which such a sequence promotes hypernegative supercoiling in cells lacking functional DNA topoisomerase I.

E. coli DM800 and ASL56 cells harboring pBR322, pBR322Bam, pBR322Δtet, and pBR322Δ188-375 were



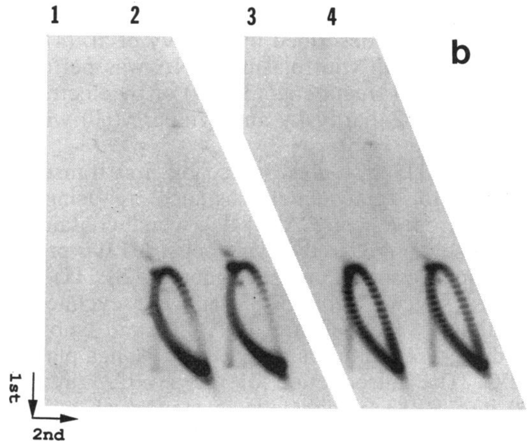
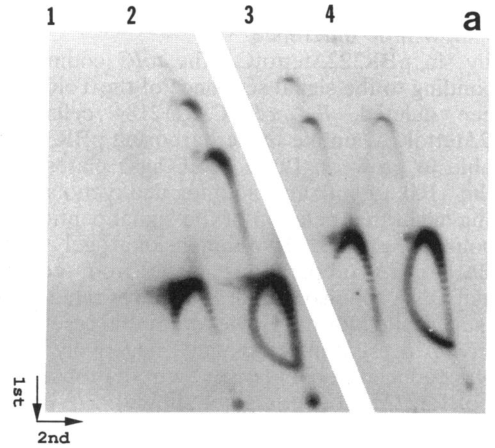
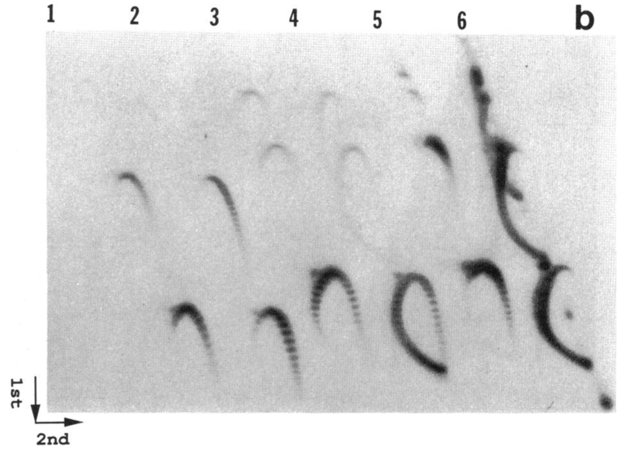


FIG. 2. (a) Two-dimensional gel electrophoresis of preparations of various pBR322 derivatives isolated from *E. coli* DM800 cells. Following isolation of DNA from the cultures, two-dimensional gel electrophoresis of the samples in 0.8% agarose slabs was carried out as described previously (35) with TAE buffer containing 7.5 and 30 μg of chloroquine disphosphate per ml in the first- (vertical) and second (horizontal)-dimension analyses, respectively. Transfer of DNA onto nylon membranes, hybridization of ^{32}P-labeled DNA probes, and autoradiography were all performed as described in Materials and Methods. Lane 1, pBR322; lane 2, pBR322Bam; lane 3, pBR322Δ188-375; lane 4, pBR322Δtet; lane 5, pBR322. During two-dimensional electrophoresis, covalently closed topoisomers of different linking numbers are separated into an arc: topoisomers of progressively higher linking numbers align clockwise around the arc, and the most highly negatively supercoiled species in each sample are present at the extreme counterclockwise end of each arc (see references 35 and 37 for discussions on the interpretation of two-dimensional electrophoretograms). In lane 4 and also in some lanes of the other figures, monomeric DNA which was irreversibly denatured during plasmid isolation is visualized as a tight spot migrating ahead of the arc of topoisomers. (b) Hypernegative supercoiling of pBR322Bam and pBR322tet1 in *E. coli* DM800. Two-dimensional gel electrophoresis of plasmid preparations was performed as described for panel a, except that the gel shown in lanes 3 and 4 contained 75 and 300 μg of chloroquine disphosphate per ml in the first- and second-dimension analyses, respectively. Lane 1, pBR322Bam; lane 2, pBR322tet1; lane 3, pBR322Bam; lane 4, pBR322tet1. (c) Use of *E. coli* ASL115 in studies of hypernegative supercoiling. Two-dimensional gel electrophoresis of plasmid preparations was performed as described for panel a. pBR322 was isolated from ASL115 cells as follows: lane 1, from cells grown at 30°C; lane 2, following temperature shift from 30 to 42°C; lane 3, from cells grown at 30°C in the presence of kanamycin (25 μg/ml), which was added at the point of division of a 30°C starter culture; lane 4, following temperature shift from 30 to 42°C, from cells grown in the presence of kanamycin (25 μg/ml) added at the point of division of the 30°C starter culture; lane 5, from cells grown at 30°C in the presence of rifampin (250 μg/ml), which was added at the point of division of a 30°C starter culture; lane 6, following temperature shift from 30 to 42°C, from cells grown in the presence of rifampin (250 μg/ml) added at the point of division of the 30°C starter culture.

grown in Luria broth containing ampicillin (100 μg/ml) at 37°C to a cell density of approximately 10^8 ml^{-1}; plasmid DNA was then prepared from each culture and analyzed by two-dimensional gel electrophoresis. As shown in Fig. 2a, highly negatively supercoiled forms of pBR322, pBR322 Bam, and pBR322Δ188-375 but not pBR322Δtet are observed in plasmid preparations made from *E. coli* DM800 Δ*topA* cells; as expected, no highly negatively supercoiled forms of any of these plasmids are observed in preparations derived from the isogenic *topA*$^+$ *gyrB225* strain *E. coli* ASL56 (data not shown). These results confirm the earlier finding of Pruss and Drlica (38) that the expression of the first 34 codons of the pBR322 Tet protein plus 88 codons of an out-of-frame *tet* segment is sufficient to effect hypernegative supercoiling of plasmid DNA in the absence of DNA topoisomerase I. The topoisomer distributions of pBR322 and pBR322Bam, as isolated from *E. coli* DM800 cells, are indistinguishable, indicating that the 5-nucleotide difference between the plasmids has no effect on their *topA*-dependent hypernegative supercoiling.

To test whether the structure of the beginning part of the *tet* mRNA or its translational product is important for the observed hypernegative supercoiling, the linking number distributions of a series of pBR322 *tet* mutants were examined. In each of these pBR322 *tet* mutant constructs 38 base pair changes in the first 100 bp of the *tet* coding sequences were introduced without altering the sequence of the protein encoded (see Materials and Methods for details). One-dimensional gel electrophoresis analysis of the topoisomer distributions of 24 such mutants, as prepared from *E. coli* DM800, revealed no significant differences compared with those of pBR322 isolated from similar cells (data not shown). Resolution of the topoisomer distribution of one such mutant, namely, pBR322tet1, by two-dimensional electrophoresis again revealed no apparent difference in comparison with pBR322 (Fig. 2b). These results strongly suggest that the





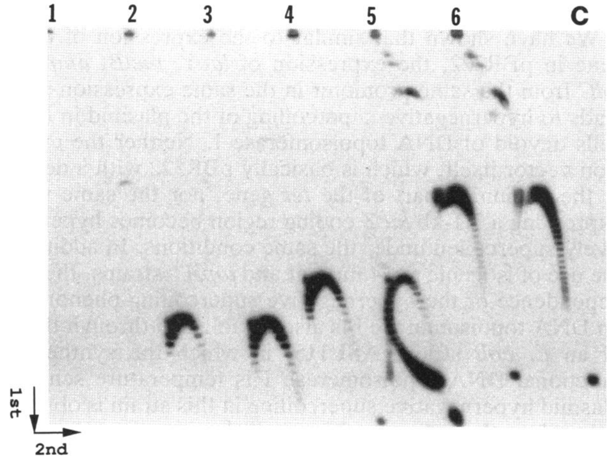


FIG. 3. Two-dimensional gel electrophoresis of plasmid preparations was performed as described in the legend to Fig. 2a. (a) Hypernegative supercoiling of pBR322ΔtetlacYΔC and pBR322ΔtetmalB in *E. coli* DM800. Lane 1, pBR322ΔtetlacYΔC isolated from *E. coli* ASL56 cells; lane 2, pBR322ΔtetlacYΔC isolated from *E. coli* DM800 cells; lane 3, pBR322ΔtetmelB isolated from *E. coli* ASL56 cells; lane 4, pBR322ΔtetmelB isolated from *E. coli* DM800 cells. (b) Hypernegative supercoiling of pBR322 and pBR322ΔtetlacY in *E. coli* ASL115 cells following thermal inhibition of DNA topoisomerase I synthesis. Lane 1, pBR322Δtet isolated from ASL115 cells grown at 30°C; lane 2, pBR322Δtet isolated from ASL115 cells following temperature shift from 30 to 42°C; lane 3, pBR322 isolated from ASL115 cells grown at 30°C; lane 4, pBR322 isolated from ASL115 cells following temperature shift from 30 to 42°C; lane 5, pBR322ΔtetlacY isolated from ASL115 cells grown at 30°C; lane 6, pBR322ΔtetlacY isolated from ASL115 cells following temperature shift from 30 to 42°C. (c) Lack of hypernegative supercoiling of pBR322ΔtetlacZ in *E. coli* DM800. Lane 1, pBR322Δtet isolated from *E. coli* ASL56 cells; lane 2, pBR322Δtet isolated from *E. coli* DM800 cells; lane 3, pBR322 isolated from *E. coli* ASL56 cells; lane 4, pBR322 isolated from *E. coli* DM800 cells; lane 5, pBR322ΔtetlacZ isolated from *E. coli* ASL56 cells; lane 6, pBR322ΔtetlacZ isolated from *E. coli* DM800 cells.

sequence of the nascent polypeptide, rather than the structure of the nascent message, is a major determinant in the hypernegative supercoiling of the *tet* plasmids.

The suitability of *E. coli* ASL115 for studies of the hypernegative supercoiling phenomenon was also demonstrated. For example, *E. coli* ASL115 harboring pBR322 was grown in Luria broth containing ampicillin (100 μg/ml) at 30°C to a cell density of approximately 5×10^7 cells ml^{-1}. Each culture was then divided into two equal portions; one half was incubated for a further hour at 42°C, and the other half was incubated for 90 min at 30°C. Plasmid DNA was then prepared from each culture and analyzed by two-dimensional electrophoresis. Figure 2c (lanes 1 and 2) shows that highly negatively supercoiled forms of pBR322 arose in *E. coli* ASL115 cells upon shifting of the temperature from 30 to 42°C. That this topological change is dependent on transcription and translation was demonstrated by the observation that the addition of rifampin (250 μg/ml, final concentration) or kanamycin (25 μg/ml, final concentration) to the cells at the time of culture division abolished the accumulation of highly negatively supercoiled forms of pBR322 at 42°C (Fig. 2c, lanes 3 to 6).

Hypernegative supercoiling of plasmids expressing integral membrane proteins. In order to test whether the transcription of genes encoding integral membrane proteins other than Tet would also cause plasmid hypernegative supercoiling in a *topA* mutant background, the coding sequences for *E. coli* lactose permease and melibiose permease, two integral cytoplasmic membrane proteins (3, 5, 6, 36, 59), were inserted into the expression vector pBR322Δtet to give pBR322ΔtetlacY and pBR322ΔtetmelB. An additional plasmid, pBR322ΔtetlacYΔC, encoding only the N-terminal half of the lactose permease protein (3) was also constructed.

As shown in Fig. 3a, both pBR322ΔtetmelB and pBR322ΔtetlacYΔC were found to exhibit hypernegative supercoiling in *E. coli* DM800 but not in ASL56. In contrast to its C-terminal deletion derivative pBR322ΔtetlacYΔC, pBR322ΔtetlacY containing the entire coding region of *lacY* cannot be stably inherited in DM800; this instability will be reported in detail elsewhere. That pBR322ΔtetlacY exhibits hypernegative supercoiling in *E. coli* cells devoid of functional DNA topoisomerase I was demonstrated by analysis of preparations made from *E. coli* ASL115 cells grown at 42°C (Fig. 3b).

As expected, the control plasmid pBR322ΔtetlacZ, in which a well-characterized cytoplasmic protein, namely, β-galactosidase, is expressed from P_{tet} in the vector pBR322Δtet, exhibits no hypernegative supercoiling in *E. coli* DM800 (Fig. 3c, lanes 5 and 6) or in *E. coli* ASL115 cells after a shift of the growth temperature from 30 to 42°C (data not shown).

Hypernegative supercoiling of plasmids expressing exported proteins. We have also examined the relation between plas-

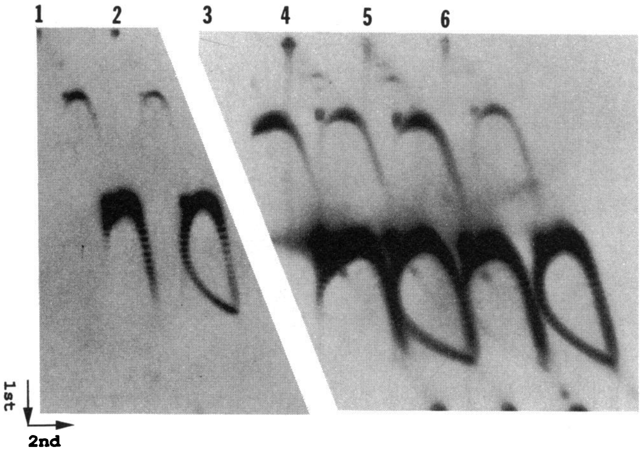


FIG. 4. Hypernegative supercoiling of pBR322ΔtetampC, pBR322ΔtetampCΔ, and pBR322ΔtettolC in *E. coli* DM800. Two-dimensional gel electrophoresis of plasmid preparations was performed as described in the legend to Fig. 2a. Lane 1, pBR322ΔtettolC isolated from *E. coli* ASL56 cells; lane 2, pBR322ΔtettolC isolated from *E. coli* DM800 cells; lane 3, pBR322ΔtetampC isolated from *E. coli* ASL56 cells; lane 4, pBR322ΔtetampC isolated from *E. coli* DM800 cells; lane 5, pBR322ΔtetampCΔ isolated from *E. coli* ASL56 cells; lane 6, pBR322ΔtetampCΔ isolated from *E. coli* DM800 cells.

mid supercoiling and the expression of polypeptides destined for export across the cytoplasmic membrane of the cell. In most cases, it is known that the physical movement of exported proteins (or domains thereof) across the cytoplasmic membrane is not temporally coupled to translational elongation (39, 40, 56) and therefore not to transcription. It seems plausible, however, that interactions between nascent polypeptides with cellular factors involved in the export process may mediate anchoring of transcripts encoding exported proteins.

Analysis of pBR322ΔtetampC, which expresses *ampC* β-lactamase for export, and pBR322ΔtettolC, which expresses the *E. coli* TolC outer membrane protein, shows that both plasmids are highly negatively supercoiled when recovered from *E. coli* DM800 Δ*topA gyrB225* but not when recovered from *E. coli* ASL56 *topA*$^+$ (Fig. 4). It had also been observed previously that highly negatively supercoiled forms of a plasmid expressing only the amino-terminal 142 amino acids of the TolC outer membrane protein were present in preparations isolated from cells lacking functional topoisomerase I (28a).

In order to determine whether the observed hypernegative supercoiling is dependent on the presence of codons for the signal peptide sequences of the AmpC and TolC proteins, two further derivatives of pBR322Δtet were constructed. In pBR322ΔtetampCΔ, the *ampC* coding sequence corresponding to the signal sequence of the AmpC preprotein has been deleted; subcloning of the *Cla*I-*Eag*I fragment of pBR322ΔtetampCΔ into pACYC184 resulted in a construct which conferred only chloramphenicol resistance on recipient cells, indicating that deletion of the signal peptide functionally abolishes export. Nevertheless, analysis of pBR322ΔtetampCΔ recovered from *E. coli* DM800 again revealed the presence of highly negatively supercoiled forms not present in preparations made from *E. coli* ASL56 (Fig. 4, lanes 5 and 6); the overall distributions of topoisomers of pBR322ΔtetampCΔ and pBR322ΔtetampC from *E. coli* DM800 show little difference.

Finally, in pBR322ΔtettolCΔ the *tolC* coding sequence corresponding to the signal sequence of the TolC preprotein has been deleted. *E. coli* CAG12184 cells harboring pBR322ΔtettolCΔ, unlike those harboring pBR322ΔtettolC, are unable to grow on Luria broth agar plates containing ampicillin (100 μg/ml) and sodium deoxycholate (0.05%), indicating again that deletion of the signal peptide functionally abolishes export. Two-dimensional gel analysis of pBR322ΔtettolCΔ preparations made from cells lacking functional DNA topoisomerase I again revealed highly negatively supercoiled forms. However, in this case the fraction of total DNA present in these highly negatively supercoiled forms was reduced in comparison with that observed in the case of pBR322ΔtettolC (data not shown).

DISCUSSION

We have shown that similar to the expression of the *tet* gene in pBR322, the expression of *lacY*, *melB*, *ampC*, or *tolC* from the same promoter in the same expression vector leads to hypernegative supercoiling of the plasmid in *E. coli* cells devoid of DNA topoisomerase I. Neither the expression vector itself, which is basically pBR322 with a deletion in the beginning part of the *tet* gene, nor the same vector expressing a 3.1-kb *lacZ* coding region becomes hypernegatively supercoiled under the same conditions. In addition to the use of isogenic *topA* mutant and *topA*$^+$ strains, the strict dependence of the hypernegative supercoiling phenomenon on DNA topoisomerase I is also established through the use of an *E. coli* strain, ASL115, in which the synthesis of functional DNA topoisomerase I is temperature sensitive; plasmid hypernegative supercoiling in this strain is observed only when the cells are devoid of the enzyme following a shift of the growth temperature to 42°C. The observation that the accumulation of highly negatively supercoiled forms of plasmids in *E. coli* ASL115 cells following a shift to 42°C is sensitive to the addition of rifampin or kanamycin to the growth media further demonstrates the requirement for transcription and translation.

Analysis of pBR322 *tet* mutants has confirmed the original finding of Pruss and Drlica (38) that expression of a chimeric polypeptide containing as few as 34 amino acids of the Tet protein is sufficient to effect hypernegative supercoiling of the plasmid. Furthermore, a total of 38 conservative changes in 29 of the degenerate codons at the beginning of *tet* has little effect on the hypernegative supercoiling of the plasmid; this finding suggests strongly that the translational product, rather than the structure of the beginning part of the *tet* message, is important in pBR322 hypernegative supercoiling.

Taken together, these results support the hypothesis that cotranscriptional membrane association can prevent the circling of a transcribing RNA polymerase around the DNA template (25, 27). Furthermore, the data presented here as well as the recent report of the hypernegative supercoiling of a plasmid expressing the *E. coli phoA* gene encoding periplasmic alkaline phosphatase (8) suggest that in bacteria, cotranscriptional anchoring of nascent polypeptides may be rather common for membrane proteins or proteins destined for export through cytoplasmic membrane. Whereas cotranscriptional membrane association appears to be one major mechanism of plasmid hypernegative supercoiling in *topA* cells, it is not the only mechanism (12, 25, 57).

Significant advances have been made in the elucidation of the biochemical pathways for the insertion of integral membrane proteins and for the export of periplasmic or outer membrane proteins; many details remain unknown, however. These pathways share certain common features but also exhibit differences depending on the particular classes of proteins involved (for recent reviews, see references 4, 43, 45, and 56). Interactions between nascent membrane or exported polypeptides and chaperone proteins, such as SecB, GroEL, DnaK, and DnaJ, are often involved; so is the participation of proteins of the export machinery, such as SecA, a peripheral membrane protein, and SecY/E, an integral membrane protein. For several small proteins, their insertion into or export across the *E. coli* cytoplasmic membrane appears to be SecA and SecY/E independent. Among the proteins studied in this work, insertion of the LacY protein, and by analogy the MelB and Tet proteins, is likely to involve SecY or a SecY-dependent function (20). Export of AmpC and TolC proteins across the cytoplasmic membrane, similar to the export of many other periplasmic or outer membrane proteins, may also involve the SecA-SecY/E pathway. It is uncertain, however, whether cotranscriptional membrane association usually involves multiprotein-mediated interactions between a nascent polypeptide and a SecY/E-type integral membrane protein.

In order to anchor the DNA template to the cytoplasmic membrane through a nascent polypeptide which does not bind DNA directly, the synthesis of the portion of the polypeptide involved in membrane association, as well as the binding of this portion to the membrane, must occur before the dissociation of its mRNA from the DNA template. Whereas in most cases the physical translocation of nascent polypeptides is not temporally coupled to translational elongation (39, 40, 56), the nascent polypeptides often interact cotranslationally with factors involved in the export process. In the case of the *E. coli lacY* product, biochemical data support a cotranslational mode of integration into the cytoplasmic membrane. Pulse-labeling studies of *E. coli* cells expressing C-terminally truncated forms of the lactose permease indicate a rapid association of nascent chains with the cytoplasmic membrane (49); N-terminal portions of LacY permease as short as 50 amino acids are able to target nascent chains to the lipid bilayer and mediate apparent attachment of the ribosomes to the membrane during chain elongation (49, 50). Studies of the synthesis of lactose permease in an *E. coli* cell-free transcription-translation system also support a model of cotranslational insertion of the nascent polypeptide into the cytoplasmic membrane. In this system, lactose permease is produced as an aggregate resistant to protease digestion and detergent solubilization, with only a small fraction of the aggregated product capable of being subsequently integrated into inside-out or inverted membrane vesicles (1). When inverted *E. coli* membrane vesicles are included in the transcription-translation system, however, the de novo-synthesized permease is found to be membrane associated in a detergent-sensitive form that yields a distinctive spectrum of proteolytic products; moreover, the membrane vesicles containing the permease synthesized in vitro acquire the capability to accumulate lactose, indicating a functional insertion of the enzyme into the membrane (1).

Studies of the cellular localization of LacZ fusion proteins bearing amino-terminal portions of the Tn*10*-specified TetA protein, which is homologous to the pBR322 *tet* product, also support the notion that amino-terminal segments as short as 38 amino acids, corresponding to the first transmembrane domain, are sufficient to confer stable membrane attachment (18). Similarly, in the case of *E. coli ampC* β-lactamase, immunoprecipitation of pulse-labeled nascent polypeptides shows that full-length newly synthesized polypeptides possessing the N-terminal signal sequence are undetectable in any of the subcellular fractions, implying that the protein is exported in a fully cotranslational manner and that the signal peptide is removed at a very early step (22).

In contrast to the cases of the LacY, Tet, and AmpC proteins discussed above, export of TEM β-lactamase encoded by the pBR322 *bla* gene occurs entirely by a posttranslational mechanism (22, 24). Thus, the plasmid hypernegative-supercoiling results reported here are entirely consistent with cotranslational membrane association of at least one polypeptide encoded by a plasmid gene. Our results also show that for the two proteins (AmpC and TolC) designated for export through the cytoplasmic membrane, the signal peptide is not necessary for effecting plasmid hypernegative supercoiling. It is possible that the expression of these deletion polypeptides effects plasmid hypernegative supercoiling by a mechanism completely different from that of their intact counterparts, for example, through aggregation of the nascent deletion polypeptides. We believe, however, that it is more likely that initial anchoring of the nascent polypeptides for export across the cytoplasmic membrane does not involve interactions between the signal peptides and their targets in the membrane.

In a rapidly dividing *E. coli* cell, hundreds of mRNA chains are being synthesized at any given time, and a significant fraction of these encode proteins designated for membrane insertion or for export through the cytoplasmic membrane. Thus, the number of points of an *E. coli* chromosome that are anchored to the cytoplasmic membrane through cotranscriptional membrane association alone is likely to be on the order of 100, which should effectively divide the chromosome into many topological domains separated by moving boundaries. The results presented here add further support to the twin-supercoiled-domain model, particularly in terms of the involvement of DNA topoisomerase I in the removal of negative supercoils behind RNA polymerases engaged in the transcription of a large fraction of the thousands of *E. coli* genes. Immunolabeling of proteins with colloidal gold particles, in combination with electron microscopic examination of thin slices of cells embedded with resins while frozen, also indicates that *E. coli* DNA topoisomerase I, as well as RNA polymerase and the HU protein, is found predominately in areas where transcription and translation are supposed to occur (10).

Whereas the present results support the notion that cotranscriptional membrane association is one mechanism of anchoring the DNA template and dividing it into multiple topological domains, other modes of template anchoring are also plausible. The type of plasmid vector used in this work, namely, pBR322Δtet, provides a means of randomly screening for genomic DNA sequence elements that can effect hypernegative supercoiling of the plasmid. The identification and characterization of such sequences should in turn provide important clues to the organization of the bacterial genome in vivo.

ACKNOWLEDGMENTS

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