Long-Distance Effect of Downstream Transcription on Activity of the Supercoiling-Sensitive leu-500 Promoter in a topA Mutant of Salmonella typhimurium

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Expression of the lacZ gene from the supercoiling-sensitive leu-500 promoter on a plasmid in topA mutant cells was stimulated by activating a divergently oriented Tac promoter, 400 bp upstream from leu-500. The stimulation was approximately threefold regardless of whether the Tac promoter drove the expression of the tet gene, whose product is membrane bound, or of the cat gene, whose product is cytosolic. Putting a second copy of the Tac promoter downstream from lacZ, approximately 3,000 bp from leu-500 in the same orientation as the latter, resulted in 30-fold increase in lacZ expression upon isopropyl-β-D-thiogalactopyranoside induction. Again, these effects were independent of the nature of the gene upstream from leu-500 (tet or cat). With both tet- and cat-harboring constructs, activation of the two Tac promoter copies caused plasmid DNA to become hypernegatively supercoiled in topA mutant cells. Thus, neither leu-500 activation nor hypernegative plasmid DNA supercoiling appears to require membrane anchoring of DNA in this system. Replacing the downstream copy of Tac with a constitutive promoter resulted in high-level lacZ expression even when the upstream copy was repressed. Under these conditions, no hypernegative DNA supercoiling was observed, indicating that the activity of plasmid-borne leu-500 in topA mutant cells does not necessarily correlate with the linking deficit of plasmid DNA. The response of the leu-500-lacZ fusion to downstream transcription provides a sensitive assay for transcriptional supercoiling in bacteria.

Translocation of RNA polymerase along the DNA template during transcription generates positive and negative supercoils (22, 41, 45). These effects can be explained if, while tracking the double helix, RNA polymerase forces DNA to spin around its axis. Axial rotation overwinds, i.e., positively supercoils, the double helix ahead in the polymerase path while underwinding the helix behind. Several lines of evidence suggest that in vivo, supercoil accumulation is buffered by the action of DNA topoisomerases. In bacteria, two distinct activities appear to be involved: DNA gyrase specifically relaxes the positive supercoils, whereas DNA topoisomerase I relaxes the negative supercoils (22, 41, 45). The roles of these two proteins were inferred from the finding that transcription causes circular DNA templates to undergo positive linking changes when gyrase is impaired and negative linking changes when topoisomerase I is impaired. The magnitude of such changes can be quite dramatic. In some plasmids obtained from strains defective in topoisomerase I activity (topA mutants), the DNA-linking deficit is so high that it can no longer be experimentally measured (1, 9, 23, 25, 29, 30, 45). This state—which was named hypernegative supercoiling (45)—was correlated with the expression of genes coding for membrane-associated proteins, such as the tetracycline resistance determinant (tet) of commonly used plasmid vectors. It was proposed that cotranscriptional anchoring of the DNA to the bacterial membrane during tet gene expression amplifies the partitioning of positive and negative supercoils (by increasing the shove for DNA axis rotation), thereby resulting in a more dramatic linking deficit once positive supercoils are selectively removed by gyrase (23–25). However, recent reports showing the formation of persistent DNA-RNA hybrids (R-loops) during transcription of the tet gene in topA mutant cells (11, 12) cast some doubt on the validity of the above proposal. Conceivably, hypernegative supercoiling might result from the gyrase-catalyzed removal of positive supercoils generated by unwinding of R-looped DNA (11, 12). Consistent with this idea, the hypernegative DNA supercoiling of plasmids expressing tet in topA mutants appears to be constrained in vivo (1).

A more reliable method for quantitating transcription-induced supercoiling is based on measuring negative superhelical tension in situ with suitable probes (4, 10, 31, 32). This method allows superhelical density to be measured at different distances from a transcription unit. Using the B- to Z-form transition of a particular DNA sequence as a sensor for negative torsional stress, Rahmouni and Wells deduced that positive supercoils produced during cat gene transcription from the Tac promoter can travel as far as 1 kb downstream from the end of this gene (32).

A different way of probing local superhelicity was provided by the leu-500 promoter of Salmonella typhimurium. This is a mutant derivative of the leucine operon promoter, which is inactivated due to an AT-to-GC transition in the −10 region (18). The defect in initiation is thought to result from an increase in the energy required for strand separation in the promoter region. This was inferred from the finding that the leu-500 defect is suppressed—that is, the promoter is reactivated—by the surpluss torsional energy caused by impairment of topoisomerase I activity (27, 33, 40). Chen et al. showed that a plasmid-borne leu-500 could become active in topA mutant cells when positioned near a divergently oriented tetA gene, provided that tetA transcription was coupled to translation and membrane anchoring of the nascent Tet polypeptide (7, 8). These were short-distance effects because leu-500 activity fell to a minimum level when the spacing between tetA and leu-500 promoters was greater than 186 bp (7, 8). The study also revealed that the tetA-mediated activation of leu-500 could be boosted by placing a third promoter on the opposite side from...
leu-500, in the same orientation as the latter (8); in this case, too, spacing was found to be critical for activation. The authors concluded that divergent transcription and membrane anchoring cooperated in concentrating negative supercoils in the leu-500 domain, thereby leading to a more efficient activation (8). The leu-500 response to both upstream and downstream transcription was independently confirmed by Tan et al. (38), who also observed the short-range nature of the effects. However, these authors did not find peptide-mediated membrane anchoring of neighboring transcripts to be necessary for leu-500 activation in their system (38). More recently, the same group reported that in plasmids carrying DNA from the chromosomal region upstream of the leu operon, leu-500 activation in topA mutant cells depended on the activity of the distant ivhIH promoter (1.9 kb away). Unlike the short-range effects, this long-range interaction appeared to be mediated by DNA sequence determinants within the intervening region (46).

The work described here originated from the idea of using the leu-500 promoter to develop an assay that might allow the identification of DNA sequences acting as barriers to supercoil relaxation. Our working hypothesis was that activation of a leu-500-lacZ fusion in topA mutant cells could be boosted upon introduction of a sequence preventing the diffusion of negative supercoils downstream from lacZ. Early tests of this hypothesis led us to characterize various aspects of leu-500 activation in topA mutant cells, including the role of downstream transcription, the contribution of membrane anchoring, and the relationships between leu-500 activity and plasmid DNA linking deficit. The results of this study and their implications are presented and discussed.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases were purchased from New England BioLabs or Boehringer Mannheim. T4 DNA ligase, Klenow enzyme, and RNase A were from Boehringer Mannheim. All enzymes were used as recommended by the manufacturer. Carbon sources, antibiotics, amino acids, chloroquine, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal), and β-galactosidase were from Sigma Chemical Co. Ultrapure DNA grade agarose was from Bio-Rad. Enzymes and chemicals. Enzymes and chemicals. Early tests of this hypothesis led us to characterize various aspects of leu-500 activation in topA mutant cells, including the role of downstream transcription, the contribution of membrane anchoring, and the relationships between leu-500 activity and plasmid DNA linking deficit. The results of this study and their implications are presented and discussed.

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Plasmid DNA preparation and chloroquine gel electrophoresis. Overnight NB-grown cultures of strain MA4719 (topA217) harboring different plasmids were diluted 100-fold in 50 ml of NB and grown at 37°C to an optical density at 600 nm of 0.35. At this point, the cells were exposed to 1 mM IPTG for 5 min. (All IPTG-dependent DNA transitions are completed within 3 min, after which the topoisomer profiles remain unchanged as long as cells grow exponentially [13; data not shown]). The cells were rapidly chilled and harvested by centrifugation, and plasmid DNA was extracted as previously described (37). DNA preparations were loaded on 1% agarose-chloroquine gel and run in Tris-phosphate-EDTA buffer containing chloroquine at 30 mg/ml. Horizontal agarose gels (14 by 11 by 0.5 cm; 60 ml) were prepared and run at room temperature with continuous buffer circulation for 22 h at 1 V cm⁻¹. The gels were treated for 30

FIG. 1. (A) Schematic representation of relevant plasmids used. Plasmids carry the coding sequences of either tet or cat genes under the control of the Tac promoter. The leu-500 promoter directs the transcription of the lacZ gene in a divergent orientation relatively to the Tac-tet or Tac-cat fusions. In some constructs, additional promoter elements are inserted in the polylinker region immediately downstream from lacZ. The orientation of these promoters is indicated by arrows. t1t2t1a and STTTh define transcription terminators (2, 6). (B) Sequences of leader regions in Tac-tet and Tac-cat gene fusions. The sequences corresponding to translation initiation triplets are underlined. In the constructs where the leader sequence includes the translatable 'cat' fragment (top sequence), the position of an in-frame stop codon is also indicated (wavy underline).
min with 1 mM MgSO\textsubscript{4} and then stained in ethidium bromide (1.5 μg ml\textsuperscript{-1}) for 4 h. Excess ethidium bromide was removed by soaking in water for 12 h with several changes of water.

**RESULTS**

**The system.** The basic features of the relevant plasmids used in this work are presented in Fig. 1; they include a lac\textsuperscript{Z} gene under the control of the leu-500 promoter and a Tac promoter approximately 400 bp upstream from leu-500 in opposite orientation to the latter. Superimposed on these constant motifs are two types of variable elements: (i) the region transcribed from Tac (hereafter called the upstream region), coding either for an integral membrane protein (the Tet protein) or for a cytosolic product (the Cat enzyme), and (ii) an additional promoter at a site immediately downstream from the lac\textsuperscript{Z} gene (hereafter called the downstream region).

Plasmids were introduced into two isogenic strains of *S. typhimurium*, a wild-type strain and a top\textsuperscript{A} mutant (topA217 [37]), both expressing the *E. coli lacI\textsuperscript{B}* repressor gene from an F\textsuperscript{+} factor. The complete IPTG dependence of the drug resistance phenotype (T\textsuperscript{cr} or C\textsuperscript{mr} [Fig. 1]) of plasmid-harboring strains suggested that they synthesized enough LacI protein to ensure the repression of Tac in multicopy. The lack of significant differences in the plasmid DNA content of cells grown in the presence or in the absence of IPTG (data not shown) ruled out possible effects of Tac/lac\textsuperscript{Z} activation on plasmid copy number. This allowed us to take β-galactosidase levels as a direct indicator of lac\textsuperscript{Z} activity.

**Role of upstream transcription.** Initially, the response of the leu-500–lac\textsuperscript{Z} fusion to upstream Tac induction was examined in the absence of downstream inserts (plasmids pBT22 and pBC23 [Fig. 1]). Results showed that lac\textsuperscript{Z} expression increased moderately but significantly (three- to fourfold) following IPTG treatment in top\textsuperscript{A} mutant cells, regardless of the cellular localization (membrane versus cytoplasm) of the product of the gene under Tac control (Table 1). No such increase was observed with pDP1, the parental plasmid lacking the leu-500 insert. When the same measurements were carried out in a top\textsuperscript{A}– genetic background, no significant change of lac\textsuperscript{Z} gene activity accompanied Tac induction (Table 2). These results strongly suggest that the increase of lac\textsuperscript{Z} expression in the pBT22- and pBC23-harboring top\textsuperscript{A} mutant strain reflects the activation of the leu-500 promoter by transcriptionally generated negative superhelical tension.

The effects of Tac-promoted transcription on plasmid DNA linking number in top\textsuperscript{A} mutant cells were examined. DNA was extracted before and after IPTG addition and subjected to agarose gel electrophoresis in the presence of chloroquine. The chloroquine concentration used for these experiments allows the separation of topoisoforms whose superhelical density (σ) is in the range between −0.060 and −0.080, with the more negatively supercoiled forms migrating faster in the gel (1; our unpublished observations). Topoisomers with |σ| ≥ 0.085 are not resolved and migrate at the front of the band distribution. The term “hypernegative supercoiling” is used throughout this work to describe such a high-mobility material specifically.

Results in Fig. 2A show that IPTG treatment causes the DNA topoisoform distributions of both pBT22 and pBC23 plasmids to shift toward lower linking values (lanes 2 to 4). Unexpectedly, in pBT22 this shift is far less dramatic than that usually associated with tet gene expression in top\textsuperscript{A} mutants (23, 29, 30, 37, 45). In particular, few hypernegatively supercoiled forms were produced, as judged from the weakness of the front band (e.g., compare Fig. 2A, lane 2, with Fig. 2B, lane 4).

Ascribing this observation to the merging and mutual annihilation of positive and negative supercoils, we speculated that the Tac-mediated stimulation of leu-500 in top\textsuperscript{A} mutant cells might be boosted by the introduction of a sequence preventing supercoil diffusion downstream from lac\textsuperscript{Z}. The possibility of scoring lac\textsuperscript{Z} expression in bacterial colonies allowed us to test the above hypothesis without any bias as to the type of sequence that would constitute a supercoil barrier.

**Role of downstream transcription.** To evaluate the frequency of sequences scoring positive in our assay, we initially tested whether any such sequence was present in pBT22. To this end, pBT22 DNA was separately digested with two restriction endonucleases: *Sna*\textsubscript{I}I, which cleaves at a unique position immediately downstream from lac\textsuperscript{Z} (Fig. 1), and *Alu*I, which cleaves pBT22 DNA 28 times. The products of *Alu*I digestion were ligated to *Sna*\textsubscript{I}I-cleaved pBT22 DNA, and the ligation products were used to transform the topA217 mutant selecting T\textsuperscript{c} on plates supplemented with IPTG and X-Gal. While the majority of transformants grew as light blue colonies, some isolates with different degrees of a darker hue were identified. Among the latter, a few colonies exhibited a distinctive deep blue. These clones (six were tested) were all found to result from the insertion of the same 104-bp *Alu*I fragment carrying the Tac promoter sequence in pBT22. In all six clones, this fragment was oriented so that transcription from the Tac promoter would be in the same direction as lac\textsuperscript{Z} transcription (plasmid pBT22 in Fig. 1). To verify these findings in the cat-expressing background, newly isolated plasmid pBT22 was digested with *Bgl*II—a treatment that released the Tac promoter on a 115-bp fragment—and the digestion products were ligated to *Bgl*II-cleaved pBC23 DNA. Transformation of the top\textsuperscript{A} mutant strain (selecting C\textsuperscript{mr} on plates supplemented with IPTG and X-Gal) led to the identification of a number of blue colonies. All the isolates tested harbored a pBC23 derivative carrying the Tac promoter fragment inserted between the tandem *Bgl*II sites downstream from lac\textsuperscript{Z}. Again, all of these clones carried the Tac promoter in the same orientation as leu-500–lac\textsuperscript{Z} (plasmid pBTC3 in Fig. 1). These results suggested that outward-directed transcription downstream from lac\textsuperscript{Z} boosted leu-500 activation in top\textsuperscript{A} mutant cells by preventing the dissipation of negative supercoils around the plasmid. Also, transcription of a limited region is apparently sufficient for such effects, since the tandem transcription termination signals of the *bla* gene (2) are expected to stop RNA polymerase less than 0.5 kb past the site of initiation.

The measurements in Table 1 show that β-galactosidase activity increases 20- to 30-fold upon induction of the divergently oriented Tac promoter elements in the resulting plasmid constructs. Again, these effects are independent of the identity of the gene upstream from leu-500 (tet or cat) and, in the case of tet, largely independent of the efficiency of Tet protein production (compare pBT22 and pBT22ΔH; the latter expresses Tet\textsuperscript{I} very poorly [see Materials and Methods and Fig. 1B]). The absence of any significant response from plasmids lacking the 162-bp leu-500 insert (pBT22ΔL and pBTC3ΔL) confirms that the boost in lac\textsuperscript{Z} transcription results entirely from the leu-500 promoter. Activation of leu-500 remains highly dependent on the presence of a top\textsuperscript{A} mutation, because only a minor increase of lac\textsuperscript{Z} expression (approximately two-fold) could be detected following IPTG treatment in the top\textsuperscript{A} cells (Table 2).

The presence of the second copy of the Tac promoter also dramatically amplifies the effects of transcription on plasmid DNA linking number in top\textsuperscript{A} mutant cells. In particular, IPTG treatment now appears to cause a hyperdeficient linking value in the majority of pBT22 and pBTC3 DNAs, as inferred from
the intensities of the front-migrating bands in Fig. 2B (lanes 4 and 6). Such findings with pBCT3 confirm that membrane anchoring of the DNA during coupled transcription and translation is not an absolute requirement for hypernegative DNA supercoiling (5, 11, 15). On the other hand, the lack of the fast-migrating band in the pBTT2D H DNA profile (Fig. 2B, lane 2) suggests that efficient expression of the gene upstream from \textit{leu-500} is critical for the supercoiling process. Examined \textit{vis-à-vis} the data in Table 1, the latter results suggest that activation of plasmid-borne \textit{leu-500} (highly efficient in pBTT2ΔH) does not necessarily correlate with plasmid DNA linking deficit (see also below).

The magnitude of the effects described above suggested that the Tac promoter downstream from lacZ played a more active role than simply preventing the diffusion of upstream-generated negative supercoils. To address this point, a new set of plasmids was constructed by replacing the downstream Tac element with an equally strong but constitutive promoter: the \textit{hisR}_{hpa} promoter of \textit{S. typhimurium} (20). When inserted in the same orientation as the \textit{leu-500}–lacZ fusion (plasmids pBTR14-9 and pBCR14-9 [Fig. 1]), \textit{hisR}_{hpa} caused significant \textit{leu-500} activation even in the absence of IPTG (Table 1). In contrast, in the opposite orientation (pBTR14-6), the \textit{hisR}_{hpa} promoter had an inhibitory effect on \textit{leu-500} activity in the absence as well as the presence of IPTG (Table 1). Once again, none of the above changes were observed in the absence of the

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\begin{tabular}{llll}
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\textbf{plasmid} & \textbf{β-galactosidase activity} & \textbf{Activation ratio} & \textbf{Arrangement} \\
\hline
\textit{-IPTG} & \textit{+IPTG} & & \\
\hline
pDP1\textsuperscript{d} & 1 & 2 & 12 & lacZ \\
pBT22 & 421 & 9 & 9 & \textit{tet} \\
pBC23 & 414 & 130 & 3 & \textit{cat} \\
pBTT2ΔL & 25 & 20 & 2 & \textit{tet} \\
pBTT2 & 3343 & 102 & 33 & lacZ \\
pBTT2ΔH & 4360 & 184 & 24 & \textit{tet} \\
pBCT3ΔL & 20 & 18 & 1 & \textit{cat} \\
pBCT3 & 4291 & 114 & 38 & \textit{cat} \\
pBTR14-9 & 3723 & 1780 & 2 & lacZ \\
pBTR14-6 & 178 & 36 & 5 & \textit{tet} \\
pBCR14-9ΔL & 30 & 32 & 1 & \textit{cat} \\
pBCR14-9 & 3351 & 1468 & 2 & \textit{cat} \\
\hline
\end{tabular}
\caption{Effect of adjacent transcription on expression of \textit{leu-500}–lacZ fusion in a \textit{topA} mutant\textsuperscript{a}}
\end{table}

\textsuperscript{a} Strain MA4719.
\textsuperscript{b} In Miller units. Values are the mean of at least three independent determinations.
\textsuperscript{c} L, T, and R stand for \textit{leu-500}, Tac, and \textit{hisR}_{hpa} promoters, respectively.
\textsuperscript{d} Parental plasmid (see Materials and Methods).
leu-500 insert (plasmid pBCR14-9DL in Table 1). These results were confirmed by cloning a number of different E. coli promoters downstream from the lacZ gene in pBT22. This work showed that the extent of leu-500 activation in topA mutant cells closely correlates with the strength of the promoter being added (data not shown).

Finally, Fig. 2C shows that the hispR orientation is also critical for attaining hypernegative DNA supercoiling in IPTG-treated topA mutant cells (compare lanes 2, 4, and 6). The fact that pBTR14-9 and pBCR14-9 express lacZ at high levels even before IPTG treatment (i.e., before becoming hypernegatively supercoiled [lanes 1 and 3]) confirms that hypernegative DNA supercoiling is not absolutely required for leu-500 activation.

### DISCUSSION

We analyzed the response of the supercoiling-sensitive leu-500 promoter to adjacent transcription in S. typhimurium. The data obtained reinforce the notion that the levels of negative superhelical tension needed for leu-500 activation are attained only in cells where topoisomerase I is defective (7, 27, 33, 40). This promoter remained largely unresponsive in wild-type cells, even in the constructs that gave high-level activation in the topA mutant background. Thus, the attractive possibility that transcriptional supercoiling plays a role in allowing genes to interact “at a distance” remains unsupported. If anything, the data collected on the leu-500 system would seem to indicate that a major role of topoisomerase I in growing cells is to suppress such types of effects.

leu-500 promoter activity was measured by using lacZ as a reporter gene. In using a functional assay, this approach differed from previous studies based on 5' end analysis of RNA transcripts (7, 8, 38, 46). Maximal lacZ expression was attained from plasmids where the leu-500–lacZ fusion was flanked by divergently oriented transcription units. This can be explained by postulating that divergent transcription drives negative supercoils into the plasmid domain limited by the divergent promoters (22). We envision the supercoiling action to result from RNA polymerase forcing rotation of the DNA axis, as depicted in the model in Fig. 3. If negative supercoils are not removed by topoisomerase I, they accumulate, reaching a level of superhelical tension suitable for leu-500 activation (Fig. 3). Similar findings were reported by other authors and similarly interpreted (8, 38). However, the striking peculiarity of our data lies in the long-range nature of the downstream effects (spanning the entire length of the lacZ coding sequence). This apparent discrepancy might relate to the fact that our assay required the spacer region between leu-500 and the downstream promoter to be completely transcribed and translated. That was not the case in previous studies, where spacing was changed by random DNA sequences likely to introduce transcriptional polarity. One might speculate that the procession of RNA polymerase molecules along the lacZ sequence provides a relay mechanism for the efficient transfer of downstream-generated superhelical tension all the way up to the leu-500 site.

Another peculiar observation in our study is that the effects of downstream transcription on lacZ expression remained substantial even when the upstream Tac promoter was repressed. Although this suggests that upstream and downstream ele-

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**Table 2. Effect of adjacent transcription on expression of leu-500–lacZ fusion in a wild-type strain**

| plasmid | β-galactosidase activity | Activation ratio | Arrangement |
|---------|--------------------------|-----------------|-------------|
|         | -IPTG | +IPTG | |
| pBT22   | 62    | 71    | 1 |
| pBC23   | 47    | 48    | 1 |
| pBTT2ΔL | 22    | 21    | 1 |
| pBTT2   | 44    | 110   | 2 |
| pBTT2ΔH | 44    | 105   | 2 |
| pBCT3ΔL | 16    | 23    | 1 |
| pBCT3   | 20    | 45    | 2 |

* Strain MA4718.
* In Miller units. Values are the mean of at least three independent determinations.
* L and T stand for leu-500 and Tac promoters, respectively.
ments act independently, the possibility that the Tac promoter contributes to the formation of a supercoiled domain even when repressed should be considered. By binding two operator sites simultaneously, the tetrameric LacI protein could promote DNA looping within the dimeric fraction of plasmids or intermolecular association of monomers (43, 44). Such protein-mediated DNA bridging was shown to severely restrict the rotational diffusion of transcription-induced DNA supercoils (43).

Our data show that the effects of adjacent transcription on leu-500 activity are virtually the same whether the gene being transcribed upstream from this promoter codes for an integral membrane protein (Tet) or for a cytosolic product (Cat). Thus, peptide-mediated anchoring of the transcription complex to the inner membrane does not appear to contribute significantly to the generation of negative superhelical tension in our system. Similar observations were made by Tan et al. (38) studying the leu-500 response to transcription from the lac promoter. In contrast, the experiments of Chen et al. convincingly showed that leu-500 activation in plasmids where the tet gene is transcribed from its normal promoter requires the cotranscriptional integration of the nascent Tet polypeptide in the bacterial membrane (8). The basis for this discrepancy remains elusive. Perhaps membrane binding is less critical when transcription initiates from promoters other than the tet promoter. The activity of the latter might be coupled to membrane insertion of the Tet protein in a way that optimizes transcriptional supercoiling. It is intriguing in this respect that expression of the tet gene from its own promoter results in high-level tetracycline-resistance—a level that is hard to reproduce even by cloning strong promoters in front of the tet coding sequence (6, 37; also see above)—and yet, the tet promoter appears to be a rather weak promoter when it is taken out of its natural context and fused to other genes (37).

Besides being dispensable for leu-500 activation, cotranscriptional membrane anchoring was not required for hypernegative plasmid DNA supercoiling in topA mutants (plasmids pBCT3 and pBCR14-9). In our system, hypernegative supercoiling required the presence of divergent transcription units initiating on the opposite ends of the leu-500–lacZ fusion. In one case, however (plasmid pBTT2AH), divergent transcription from two Tac promoters allowed maximal leu-500–lacZ activation but failed to promote hypernegative DNA supercoiling. The Tac-tet gene fusion in pBTT2AH expresses the resistance determinant very inefficiently due to an unknown defect that causes the tet mRNA to be rapidly degraded or prematurely terminated (37). Whatever the nature of this defect, the behavior of pBTT2AH suggests that the mere "functioning" of the flanking promoters is sufficient for leu-500 activation whereas production of an RNA transcript in the region upstream from leu-500 is required to attain hypernegative plasmid DNA supercoiling. Clearly, this would be consistent with the idea that hypernegative supercoiling reflects RNA-DNA hybrid formation during transcription (11, 12). Also in agreement with the data presented here are the results of an independent study in which we found no correlation between the production of unconstrained negative DNA supercoils (monitored by chemical probing) and the increase in DNA linking deficit during transcription in topA mutants (1).

Overall, the results presented here cast doubt on the rele-

![FIG. 2. Effect of Tac promoter activation on the topoisomer profiles of plasmid DNA in topA mutant cells. DNA was extracted from exponentially growing cells of strain MA4719 (topA217) as described in Materials and Methods and loaded onto a 1% agarose gel containing 30 μg of chloroquine ml−1. At this chloroquine concentration, topoisomers with a smaller linking number migrate faster in the gel. (A) Lanes 1 and 2, pBT22 (Tc r) DNA extracted before (lane 1) and 5 min after (lane 2) addition of 1 mM IPTG. Lanes 3 and 4, pBC23 (Cm r) DNA extracted before (lane 3) and 5 min after (lane 4) addition of 1 mM IPTG. (B) Lanes 1 and 2, pBT22ΔH DNA extracted before (lane 1) and 5 min after (lane 2) addition of 1 mM IPTG. Lanes 3 and 4, pBT22 DNA extracted before (lane 3) and 5 min after (lane 4) addition of 1 mM IPTG. Lanes 5 and 6, pBCT3 DNA extracted before (lane 5) and 5 min after (lane 6) addition of 1 mM IPTG. The fastest-migrating bands in lanes 4 and 6 correspond to unresolved, highly negatively supercoiled DNA topoisomers. (C) Lanes 1 and 2, pBTR14-9 DNA extracted before (lane 1) and 5 min after (lane 2) addition of 1 mM IPTG. Lanes 3 and 4, pBCR14-9 DNA extracted before (lane 3) and 5 min after (lane 4) addition of 1 mM IPTG. Lanes 5 and 6, pBTR14-6 DNA extracted before (lane 5) and 5 min after (lane 6) addition of 1 mM IPTG. The fastest-migrating bands in lanes 2 and 4 correspond to unresolved, highly negatively supercoiled DNA topoisomers.
average DNA linking deficit is set by gyrase activity. T, Tac promoter; L, leu-500–lacZ in plasmid. IPTG, plasmid DNAsupercoiling is un perturbed. The apical position of RNA polymerase through the elongation cycle prevents circularization of the transcription complex around the template, thus making it unnecessary to postulate a similar role for membrane anchoring. T, Tac promoter; L, leu-500 promoter.

vance of cotranscriptional membrane anchoring in the generation of unconstrained supercoils. The immediate implication is that the ability to force rotation of the DNA axis during elongation is intrinsic to the mechanism of action of RNA polymerase. Consistent with this interpretation, electron microscopic observations showed that RNA polymerase maintains an apical position that prevents circularization around the DNA template during transcription of supercoiled DNA (39) (Fig. 3).

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