Activation of the *leu-500* Promoter: A Topological Domain Generated by Divergent Transcription in a Plasmid†

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ABSTRACT: The *Salmonella typhimurium leu-500* promoter is active only in *topA* strains. In an earlier study (Chen, D., Bowater, R., Dorman, C., & Lilley, D. M. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8784–8788), we showed that the activity of this promoter on a circular plasmid is a function of the transcription and translation of an adjacent *tetA* gene, and we suggested that the effect arises because of increased local negative superhelix density due to transcription (Liu, L. F., & Wang, J. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7024–7027) initiated at the *tetA* promoter. In this study we show that translation of the 5′ (N-terminal) section of *tetA* is required for activity of the *leu-500* promoter, consistent with a requirement for membrane association of TetA. We have also shown the importance of a second transcription unit, the ampicillin resistance gene *bla*, in the activation of the *leu-500* promoter. Thus the activity of the *leu-500* promoter was reduced by partial deletion or premature termination of *bla* and was increased when the transcription of *bla* was boosted by the insertion of the stronger *tac* promoter. However, even in the latter situation the role of the *tetA* gene is dominant, and deletion of the *tetA* gene reduced activity of the *leu-500* promoter to very low levels. These results suggest the existence of a topological domain defined by the divergent *bla* and *tetA* transcription units. Membrane insertion at *tetA* is essential to provide an anchorage point. Insertion of random DNA sequences within the *bla*-to-*tetA* domain resulted in a reduction in initiation of transcription at the *leu-500* promoter, whereas insertion outside the domain had almost no effect. These observations are consistent with activation of the *leu-500* promoter by negative supercoiling in the *bla*-to-*tetA* domain, the steady-state superhelix density of which is a function of the relative rates of induction by transcription and relaxation, and the length of DNA between the divergent genes.

Transcription and DNA supercoiling are closely linked. Many promoters are thought to respond to changes in DNA supercoiling (Pruss & Drlica, 1989), and transcription may itself affect the local level of superhelical tension in the template DNA (Liu & Wang, 1987; Pruss & Drlica, 1986; Tsao et al., 1989; Wu et al., 1988). We recently discussed the case of the *Salmonella typhimurium leu-500* promoter, which appears to illustrate both of these phenomena (Chen et al., 1992; Lilley & Higgins, 1991). Our results have suggested that this promoter is activated on a circular plasmid by negative DNA supercoiling arising from the transcription of an adjacent, divergent gene.

leu-500 (Mukai & Margolin, 1963) is a chromosomal A-to-G mutation in the −10 region (Gemmill et al., 1984) of the *S. typhimurium* leucine biosynthetic operon that is normally transcriptionally inactive but which becomes activated in strains carrying null mutations in the structural gene for topoisomerase I (*topA*) (Dubnau & Margolin, 1972; Margolin et al., 1985; Pruss & Drlica, 1985; Trucksis et al., 1981). This suggests (Pruss & Drlica, 1985; Smith, 1981) that the *leu-500* promoter is being activated by the elevated level of negative supercoiling that arises in *topA S. typhimurium* strains (Richardson et al., 1984); however there are a number of inconsistencies with this model. First, we observed that the suppression of *leu-500* correlated exactly with the inactivation of *topA* rather than with the net level of negative supercoiling, and second, we could not observe the suppression when the *leu-500* promoter was carried on a circular plasmid (Richardson et al., 1988). To account for these observations, we proposed a new model in which the *leu-500* promoter was activated by the domain of negative supercoiling arising from transcription of a nearby promoter (Lilley & Higgins, 1991).

We showed that the *leu-500* promoter became selectively activated on a circular plasmid in a Δ*topA* host when adjacent to the tetracycline resistance gene *tetA* and that this activation required both transcription and translation of the *tetA* gene (Chen et al., 1992). Transcription of the *tetA* gene has been associated with changes of topoisomer distribution in plasmids (Pruss & Drlica, 1986), and it was shown that translation as well as transcription was important in this effect (Lodge et al., 1989). Plasmid oversupercoiling occurs rapidly after the induction of expression of *tetA* (Cook et al., 1992).

We proposed a mechanism for the activation of the *leu-500* promoter on the plasmid (Chen et al., 1992). A domain of negative supercoiling may be generated due to transcription of the *tetA* gene, the steady-state level of which is normally reduced by two mechanisms. Enzymatic relaxation of negative supercoiling by topoisomerase I is reduced, if not prevented, in a *topA* host. However, it is still possible to reduce local superhelix density by diffusion and cancellation of positive and negative supercoiling around the circular DNA molecule. It seems probable that this reduction is the result of anchorage of the transcribing complex due to insertion of the nascent TetA peptide in the inner membrane of the cell. Thus activation of the *leu-500* promoter on a circular plasmid requires both the expression of *tetA* and the inactivation of *topA*.

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This mechanism leaves a number of open questions, which we now address. Although we have demonstrated the requirement for translation of TetA for the activation of plasmid-borne *leu-500*, we have not demonstrated a requirement for membrane insertion. Secreted and membrane-associated proteins are generally directed to the membrane by hydrophobic N-terminal sections of peptide, and removal of this region should interfere with the vectorial process. Thus N-terminal deletions should reduce the anchorage of the plasmid to the membrane and reduce the initiation of transcription at the *leu-500* promoter as a consequence.

A second question goes deeper to the heart of the proposed mechanism. Although insertion of the TetA protein in the membrane should anchor the transcribing RNA polymerase to the membrane, it would be predicted that superhelical diffusion could still be possible around the opposite side of the plasmid that carries the ampicillin resistance gene *bla* and the replication origin. We have wondered what features in this region of the plasmid might hinder the diffusional process, such as a second transcription unit or other barriers such as large DNA–protein complexes. We have focused our attention on the expression of the *bla* gene.

We find that the activation of the *leu-500* promoter on a circular plasmid is consistent with its location in a small topological domain that is defined by the divergent *bla* and *tetA* genes and that the transcription, translation, and membrane insertion of one protein product is essential in the activation.

MATERIALS AND METHODS

Growth of Bacterial Strains. CH582 is a Δ*topA2726 leu-500 ara-9* derivative of *S. typhimurium* LT2 (Richardson et al., 1984). Bacteria were cultured at 37 °C with aeration in LB medium or grown on 1.2% LB agar plates. CH582 was grown in leucine-rich LB medium. Media were supplemented with antibiotics as required; ampicillin at 50 μg/mL, tetracycline at 10 μg/mL, or kanamycin at 50 μg/mL. Plasmids were transformed into CH582 by the calcium chloride procedure (Cohen et al., 1972) and were extracted from cells by the SDS–alkali method (Birnboim & Doly, 1979). All DNA sequences were checked by chain termination methods (Sanger et al., 1977). Strains containing plasmids carrying the *tac* promoter were cotransformed with plasmid pLacI^Q (Kan^r) to provide *lac* repressor in *trans*. For induction of the *tac* promoter, IPTG was added to a final concentration of 1 mM 15 min before the culture reached midlogarithmic phase.

Extraction and Analysis of Cellular RNA. RNA was prepared from freshly inoculated cultures at midlogarithmic phase. Culture samples of 200 μL were diluted with an equal volume of 20 mM sodium acetate (pH 5.2), 2% SDS, and 0.3 M sucrose and placed in a boiling water bath for 1 min. This was phenol extracted, and nucleic acids were precipitated with ethanol. After the addition of 0.2 pmol of the appropriate [5′-^{32}P]-labeled DNA primer, the sample was heated to 90 °C in 4.5 μL of 50 mM Tris (pH 8.0) and 50 mM KCl and rapidly cooled. Twenty-five units of RNasin (0.5 μL) were added, and the solution was incubated at 43 °C for 20 min before addition to 12 μL of 70 mM Tris (pH 8.0), 70 mM KCl, 15 mM $MgCl_2$, 15 mM dithiothreitol, and 1.3 mM dNTPs containing 50 units of MMLV reverse transcriptase (Superscript Plus, BRL) and subsequent incubation at 42 °C for 2 h. Transcripts were electrophoresed in 6% polyacrylamide in 90 mM Tris borate (pH 8.3) and 10 mM EDTA (TBE buffer) containing 7 M urea, adjacent to sequence markers generated by dideoxy sequence reactions that used the same primer. Radioactive fragments on dried gels were observed by autoradiography at −70 °C with intensifier screens or with storage phosphor screens and a 400S phosphorimager (Molecular Dynamics). Quantitation was performed on the phosphorimage.

Two primers were employed for the analysis of RNA transcripts. The oligonucleotide 5′-CCTGACGTCTAAGAAACC-3′, which hybridizes to vector sequences between the *Eco*RI site and the *bla* gene, was used for all the plasmids based directly on pLEU500Tc. For pLEU500 P$_{tac}$*bla* and its derivatives, an alternative primer was used to avoid priming cDNA synthesis from transcripts arising from the *tac* promoter. This primer had the sequence 5′-CCGCGCACAATAAATGCG-3′ and hybridized within *S. typhimurium* sequences between the *tac* and *leu-500* promoters. In principle, this primer would also detect transcripts from the chromosomal copy of *leu*, but transcription from the chromosomal gene was repressed by growth in leucine-rich medium; in control experiments using RNA extracted from CH582 (containing no plasmid) under these conditions, we detected no transcription of *leu* sequences.

Plasmid Construction. All the plasmids employed in these studies are summarized in Table I.

(*1*) *Derivatives of pLEU500Tc.* pLEU500 Δ(2–30)*tetA*. This was constructed by replacing the fragment of pLEU500Tc between the *Hin*dIII and *Nhe*I sites with synthetic oligonucleotides corresponding to the *tetA* sequence lacking the 87-bp encoding amino acids 2–30 of TetA.

pLEU500 Δ*bla*. pLEU500Tc was cleaved at the *Ssp*I and the *Sca*I sites, and the blunt-ended fragment was ligated with T4 DNA ligase.

pLEU500 *bla* ter *Eco*57 and pLEU500 *bla* ter *Sca*I. Synthetic self-complementary oligonucleotides encoding translation terminators were introduced into the *Eco*57 (CTAGCTAGCTAGCG) or the *Sca*I (CTAGCTAGCTAG) sites within the *bla* gene of pLEU500Tc.

pLEU500P$_{tac}$*bla*. The complementary oligonucleotides 5′-AATTCTGTTGACAATTATTCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACA-3′ and 5′-AATTTGTGTGAAATTGTTATCCGCTCACAATTCCACACATTATACGAGCCGATGATTAATTGTCAACAG-3′ (containing the *tac* promoter and its operator) were ligated into the *Eco*RI site of pLEU500Tc. Restriction enzyme digestion and DNA sequencing were used to select the leftward orientation of the *tac* promoter.

pLEU500 *Xba*. The complementary synthetic oligonucleotides 5′-AGCTCTAGACTGTCGCCATTGCATTTTGTTGACGATATCCGATA-3′ and 5′-AGCTTATCGGATATCGTCAACAAAATGCAATGGCGACAGTCTAG-3′ were inserted into the *Hin*dIII site of pLEU500Tc. This resulted in a net insertion of 44 bp between the *tetA* gene and the *leu-500* promoter. The sequence was chosen to reconstruct the upstream region of the *tetA* gene and to place an *Xba*I site upstream of the *tetA* promoter.

pLEU500 *Xba* 186, pLEU500 *Xba* 466, pLEU500 *Xba* 787, and pLEU500 *Xba* 1530. These plasmids were constructed by insertion of additional DNA sequences into the *Xba*I site of pLEU500 *Xba*. Fragments of 142, 422, and 743 bp were excised from pLEU500Tc species in which termination codons had been introduced into the *tetA* gene (Chen et al., 1992) and used to construct pLEU500 *Xba* 186, pLEU500 *Xba* 466, and pLEU500 *Xba* 787, respectively. Two copies of the 743-bp fragment were introduced to generate pLEU500 *Xba* 1530.

Table I: Summary of the Plasmids Constructed in the Course of These Studies[a]

Plasmids Derived from pLEU500Tc	
pLEU500Tc	parent plasmid (see Figure 1) (Chen et al., 1992)
pLEU500 Δ(2–30) *tetA*	coding for amino acids 2–30 deleted from *tetA*
pLEU500 Δ*bla*	deletion of *bla* between *Ssp*I and *Sca*I sites
pLEU500 *bla* ter *Eco*57	termination codon in *bla* at *Eco*57 site (12 aa of Bla)
pLEU500 *bla* ter *Sca*I	termination codon in *bla* at *Sca*I site (80 aa of Bla)
pLEU500P$_{tac}$*bla*	*tac* promoter inserted at *Eco*RI site (see Figure 4A)
pLEU500 *Xba*	44 bp inserted into *Hin*dIII site to create *Xba*I site
pLEU500 *Xba* 186	pLEU500 Xba with 142 bp inserted into *Xba*I site
pLEU500 *Xba* 466	pLEU500 Xba with 422 bp inserted into *Xba*I site
pLEU500 *Xba* 787	pLEU500 Xba with 743 bp inserted into *Xba*I site
pLEu500 *Xba* 1530	pLEU500 Xba with 1486 bp inserted into *Xba*I site
pLEU500 *Eco* 754	pLEU500Tc with 754 bp inserted into *Eco*RI site
pLEU500 *Eco* 1497	pLEU500Tc with 1497 bp inserted into *Eco*RI site
pLEU500 *Ava* 754	pLEU500Tc with 754 bp inserted into *Ava*I site
Plasmids Derived from pLEU500P$_{tac}$*bla*	
pLEU500P$_{tac}$Δ*bla*	deletion of *bla* between *Ssp*I and *Sca*I sites
pLEU500P$_{tac}$*bla* ter *Eco*57	termination codon in *bla* at *Eco*57 site
pLEU500P$_{tac}$*bla* ter *Sca*I	termination codon in *bla* at *Sca*I site
pLEU500P$_{tac}$*bla*. Δ*tetA*	deletion of 166-bp *Eco*RV fragment (*tetA* promoter)
pLEU500P$_{tac}$*bla.tetA* ter *Nhe*I	termination codon in *tetA* at *Nhe*I site (48 aa of TetA)
pLEU500P$_{tac}$*bla.tetA* ter *Bam*HI	termination codon in *tetA* at *Bam*HI site (97 aa of TetA)
pLEU500P$_{tac}$*bla.tetA* ter *Sal*I	termination codon in *tetA* at *Sal*I site (187 aa of TetA)
pLEU500P$_{tac}$*bla.tetA* ter *Nru*I	termination codon in *tetA* at *Nru*I site (296 aa of TetA)
pLEU500P$_{tac}$*bla Xba*	44 bp inserted into *Hin*dIII site to create *Xba*I site
pLEU500P$_{tac}$*bla Xba* 186	pLEU500P$_{tac}$*bla* Xba with 142 bp inserted into *Xba*I site
pLEU500P$_{tac}$*bla Xba* 466	pLEU500P$_{tac}$*bla* Xba with 422 bp inserted into *Xba*I site
pLEU500P$_{tac}$*bla Xba* 787	pLEU500P$_{tac}$*bla* Xba with 743 bp inserted into *Xba*I site
pLEU500P$_{tac}$*bla Xba* 1530	pLEU500P$_{tac}$*bla* Xba with 1486 bp inserted into *Xba*I site

[a] See Materials and Methods for the full details of the constructions.

pLEU500 *Eco* 754 and pLEU500 *Eco* 1497. The synthetic oligonucleotides 5′-AATTCTCTAGA-3′ and 5′-AATTTCTAGAG-3′ were ligated into the *Eco*RI site of pLEU500Tc to generate an *Xba*I site. The 743- or 1486-bp fragments described above were ligated into the *Xba*I site.

pLEU500 *Ava* 754. The synthetic oligonucleotides 5′-TCGGGTCTAGA-3′ and 5′-CCGATCTAGAC-3′ were ligated into the *Ava*I site of pLEU500Tc, and the 743-bp fragment was ligated into the *Xba*I site contained within the synthetic sequence.

(*2*) *Derivatives of pLEU500 P$_{tac}$bla.* pLEU500P$_{tac}$Δ*bla.* pLEU500P$_{tac}$*bla* was cleaved at the *Ssp*I and the *Sca*I sites, and the blunt-ended fragment was ligated with T4 DNA ligase.

pLEU500P$_{tac}$*bla* ter *Eco*57 and pLEU500P$_{tac}$*bla* ter *Sca*I. Synthetic self-complementary oligonucleotides encoding translation terminators were introduced into the *Eco*57 (CTAGCTAGCTAGCG) or the *Sca*I (CTAGCTAGCTAG) sites within the *bla* gene of pLEU500P$_{tac}$*bla*.

pLEU500P$_{tac}$*bla.*Δ*tetA*. The 166-bp *Eco*RV fragment containing the *tetA* and *antitet* promoters was excised from pLEU500P$_{tac}$*bla*, and the parent molecule was recircularized with T4 DNA ligase.

pLEU500P$_{tac}$*bla.tetA* ter *Nhe*I, pLEU500P$_{tac}$*bla.tetA* ter *Bam*HI, pLEU500P$_{tac}$*bla.tetA* ter *Sal*I, and pLEU500P$_{tac}$*bla.tetA* ter *Nru*I. Synthetic oligonucleotide translation terminators were inserted into the *tetA* gene of pLEU500P$_{tac}$*bla* at the *Nhe*I, the *Bam*HI, the *Sal*I, or the *Nru*I sites. These were exactly equivalent to the series of plasmids based on pLEU500Tc that we described previously (Chen et al., 1992).

pLEU500P$_{tac}$*bla Xba.* The construction was exactly equivalent to that of pLEU500Tc *Xba* from pLEU500Tc described above.

pLEU500P$_{tac}$*bla Xba* 186, pLEU500P$_{tac}$*bla Xba* 466, pLEU500P$_{tac}$*bla Xba* 787, and pLEU500P$_{tac}$*bla Xba* 1530. The construction of these plasmids was exactly equivalent to that of pLEU500Tc *Xba* 186, etc. from pLEU500Tc described above.

RESULTS

Deletion of the N-Terminal Peptide of TetA Reduces the Activity of the leu-500 Promoter. We have previously demonstrated the activation of a plasmid-borne *leu-500* promoter in Δ*topA* strains of *S. typhimurium* when it is inserted upstream of the tetracycline resistance gene *tetA* (Chen et al., 1992). This was achieved by using the plasmid pLEU500Tc and derivatives thereof (Figure 1). We showed that both transcription and translation of *tetA* were required for the function of the *leu-500* promoter (but *not* for that of the *antitet* promoter) and suggested that the activation was related to membrane insertion of the TetA protein. To test this hypothesis, we decided to modify the *tetA* gene to generate a truncated form of the TetA protein lacking amino acids 2–30; this hydrophobic section of peptide constitutes the first transmembrane segment (Allard & Bertrand, 1992; Eckert & Beck, 1989) and is likely to be critical in the membrane insertion process.

Initiation of transcription of the *leu-500* promoter was analyzed by extraction of RNA and reverse transcription, with use of a primer that is specific for transcription from the appropriate strand of the plasmid DNA (see Figure 1). Transcription initiating at either the *leu-500* or the *antitet*(*tetR*) promoter generates cDNA species of known size. We compared transcription arising from the plasmid-borne *leu-500* promoters in CH582 (Δ*topA*) coupled to either the normal *tetA* gene or the *tetA* gene carrying the 5′ deletion (Figure 2). Deletion of the N-terminus of the protein was found to reduce transcription initiation at the *leu-500* promoter by a significant amount, while that at the control *antitet* promoter was unaffected. Although the deletion only reduced the calculated molecular weight of the translated TetA protein by a factor of 0.93, it reduced transcription from the *leu-500* promoter to less than 25% of that observed when

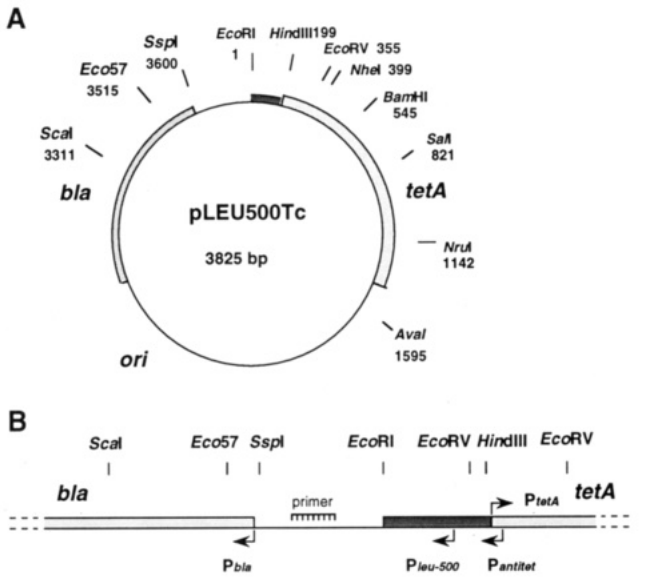

FIGURE 1: Plasmid map of pLEU500Tc which contains the region of *S. typhimurium* DNA containing the *leu-500* promoter adjacent to the tetracycline resistance gene *tetA* (Chen et al., 1992). This plasmid is the starting point for all the plasmid constructs used in these studies. The *S. typhimurium* fragment containing the *leu-500* promoter (stippled, dark) was cloned immediately adjacent to the *tetA* gene (stippled, light). Restriction sites used in these experiments are shown. The linear map shows the region between the *bla* and *tetA* transcription units, showing the position and orientation of the *leu-500*, *bla*, *tetA*, and *antitet* promoters. The *antitet* promoter is the promoter of the absent *tetR* gene; we have observed that its activity is not dependent on either *topA* or *tetA* function, and it serves as an internal control of the basal level of RNA initiation in many experiments. Note the location of the primer used to study initiation of transcription in plasmids based on pLEU500Tc; this lies outside the *S. typhimurium* DNA, in vector sequences, and therefore detects transcription arising only from the plasmid-borne *leu-500* promoter and the *antitet* promoter. (A) Complete circular map of pLEU500Tc. (B) Expanded detail of the region of pLEU500Tc between the *bla* and *tetA* genes. The *S. typhimurium* sequences are shown dark stippled.

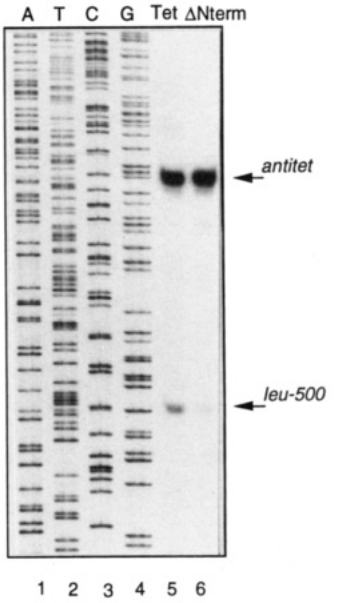

FIGURE 2: Reduction in transcriptional activity of the *leu-500* promoter in a Δ*topA* host, which results from deletion of the N-terminal region of TetA. A plasmid (pLEU500Δ(2–30) *tetA*) was constructed from pLEU500Tc such that *tetA* encoded a modified TetA protein lacking amino acids 2–30, i.e., expressing a polypeptide comprising the initiator methionine fused to amino acids 31 to the C-terminus. Run-off cDNA transcripts were made by reverse transcription from RNA extracted from *S. typhimurium* CH582 (Δ*topA*), transformed with either pLEU500Tc (track 5) or pLEU500Δ(2–30)*tetA* (track 6). Transcription initiating at the *leu-500* promoter generates the band indicated by the lower arrow shown on the right, while transcription initiating at the *antitet* promoter generates the band indicated by the upper arrow. Note the reduction in intensity of the band corresponding to initiation of transcription at the *leu-500* promoter in the plasmid lacking the N-terminal section of *tetA*, while initiation at the *antitet* promoter is essentially unaffected by this change. Tracks 1–4 contain sequence markers generated by dideoxy sequencing using the same primer.

the unmodified *tetA* gene was present. The modified sequence lies entirely outside the region transcribed from the *leu-500* promoter and could not, therefore, affect mRNA stability; it must, therefore, reflect relative initiation at the *leu-500* promoter. This strongly suggests that TetA membrane insertion plays an important role in the activation of the *leu-500* promoter in the Δ*topA* strain.

Deletion of the bla Gene Reduces the Activity of the leu-500 Promoter. As we discussed in the introduction section, we reasoned that the model for the activation of the *leu-500* promoter might require the existence of a second topological barrier in the circular plasmid. The ampicillin resistance gene *bla*, which is divergent with respect to *tetA*, generates a protein (β-lactamase) that is exported into the cell periplasm. Potentially it might serve as a topological barrier or as the generator of additional negative supercoiling. Beginning with the plasmid pLEU500Tc, we made a deletion of the 5′ section of the *bla* gene between the *Ssp*I and *Sca*I sites; this removes 30% of the N-terminal coding sequence. Initiation of transcription at the *leu-500* promoter in CH582 (*topA*) was analyzed as above (Figure 3). Deletion of part of the *bla* gene resulted in a substantial reduction in initiation at the *leu-500* promoter.

Termination of β-Lactamase Translation Reduces the Activity of the leu-500 Promoter. As a further test of the importance of *bla* expression to the activation of the *leu-500* promoter, we examined the effect of premature termination of translation of the β-lactamase protein. Termination codons were introduced into the *bla* coding sequences by ligation of oligonucleotides into either the *Eco*57 or the *Sca*I site, generating peptides shortened from 263 amino acids to 12 or 80 amino acids, respectively. The effect upon initiation of transcription at the *leu-500* promoter in CH582 (*topA*) was analyzed as before (Figure 3); both resulted in a marked reduction in initiation of transcription at the *leu-500* promoter. Thus translation of *bla* plays a role in the Δ*topA*-dependent activation of the *leu-500* promoter in pLEU500Tc.

leu-500 Promoter Expression Is Increased by Insertion of a Stronger Promoter behind the bla Gene. Since a lowering in the expression of the *bla* gene results in a reduction in initiation of transcription of the *leu-500* promoter, we tested the effect of increasing the level of *bla* expression by insertion of the stronger [by a factor of approximately 17 (Deuschle et al., 1986)] *tac* promoter (De Boer et al., 1983) in the *Eco*RI site of pLEU500Tc to create plasmid pLEU500P_{tac}*bla* (Figure 4). Increasing transcription of the *bla* gene increased the level of initiation at the *leu-500* promoter by 1 order of magnitude (varying between 5- and 10-fold in different experiments). By contrast, when the *tac* promoter was placed in the *Eco*RI site with the opposite orientation (i.e., transcribing toward *tetA*), we could detect no initiation of RNA synthesis at the *leu-500* promoter (data not shown). Thus the level of *leu-500* promoter activation can be modulated in *both* directions by manipulating the expression of *bla*.

We wondered how the *leu-500* promoter would respond to deletion and termination of the *bla* gene in the case in which

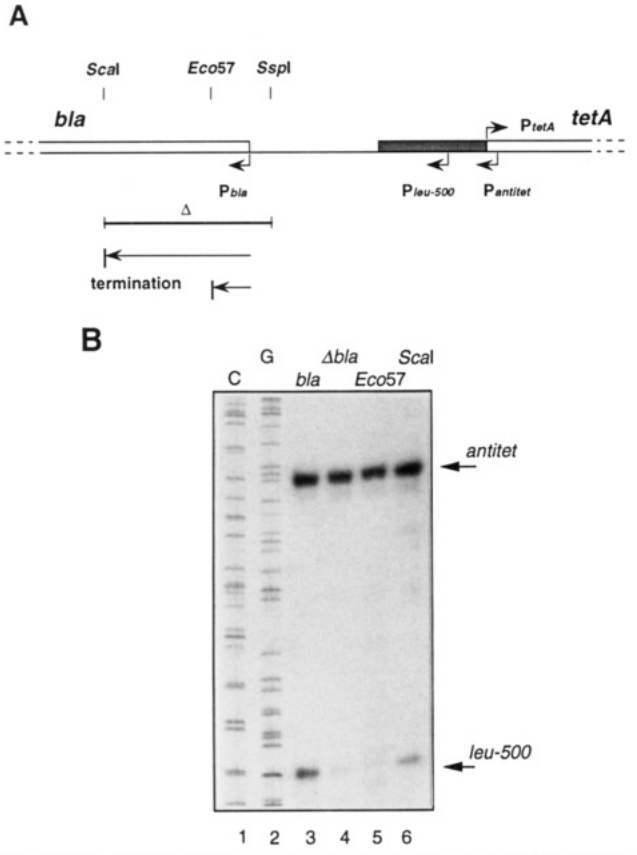

FIGURE 3: Effect of the ampicillin resistance gene (*bla*) on the activation of the *leu-500* promoter. (A) Map of the relevant part of pLEU500Tc; showing the modifications made to the plasmid in and around the *bla* gene. A deletion was made by excision of the region between the *Ssp*I and *Sca*I sites; this removes the entire 5′ end of *bla*. Translation termination codons were introduced into the *Eco*57 and *Sca*I sites in separate constructions. (B) Autoradiograph of sequencing gel showing results of reverse transcription of cellular RNA extracted from CH582. Transcription initiating at the *leu-500* promoter generates the band indicated by the lower arrow shown on the right, while transcription initiating at the *antitet* promoter generates the band indicated by the upper arrow. Tracks 1 and 2 contain C and G dideoxy sequencing markers, respectively. Track 3 shows cDNA derived from unmodified pLEU500Tc. Track 4 shows the result of deletion of *bla*, while tracks 5 and 6 show the result of termination of *bla* translation at the *Eco*57 and *Sca*I sites, respectively. Note the reduced level of initiation of transcription at the *leu-500* promoter evident as a result of modifications to the *bla* gene.

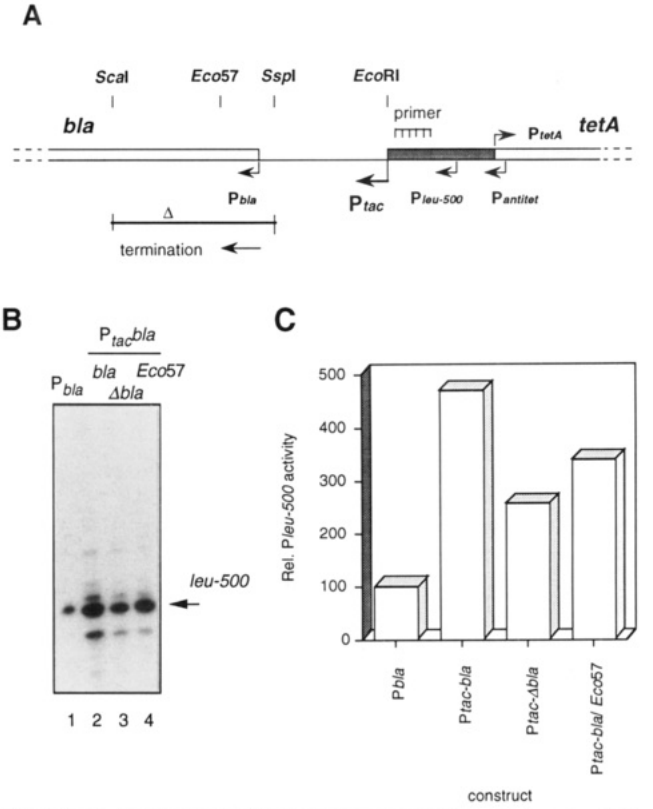

FIGURE 4: Increased initiation of transcription at the *leu-500* promoter, which results from increased transcription of *bla*. (A) Map of the relevant part of pLEU500P$_{tac}$*bla*, showing the location of the inserted *tac* promoter and the modifications made to the plasmid in and around the *bla* gene. A deletion was made by excision of the region between the *Ssp*I and *Sca*I sites, removing the entire 5′ end of *bla*. A termination codon was introduced into the *Eco*57 site. Note that in all experiments using plasmids based on pLEU500P$_{tac}$-*bla*, we have used a different primer for the analysis of RNA initiated *in vivo*, one that anneals within the *S. typhimurium* sequences. Thus the pattern of cDNA bands appears slightly different from those in which the other primer was used. (B) Autoradiograph of sequencing gel showing results of reverse transcription of cellular RNA extracted from CH582. Transcription initiating at the *leu-500* promoter generates the band indicated by the arrow shown on the right. Track 1 contains cDNA generated from cellular RNA extracted from cells containing pLEU500Tc, i.e., the plasmid lacking the *tac* promoter. Track 2 contains cDNA generated from cellular RNA extracted from cells containing pLEU500P$_{tac}$*bla*. Tracks 3 and 4 contain cDNA generated from cellular RNA extracted from cells containing pLEU500P$_{tac}$*bla* modified by deletion and translation termination, respectively, of *bla*. (C) The data of (B) were quantified by phosphorimaging and are presented as a histogram showing relative degrees of initiation at the *leu-500* promoter in the different constructs. Note the large increase in the activity of the *leu-500* promoter with the introduction of the *tac* promoter and that this is not fully negated by either deletion or termination of *bla*.

transcription was initiated at the *tac* promoter. We therefore introduced the same alterations to *bla* in pLEU500P$_{tac}$*bla* that we discussed above in the context of the normal *bla* promoter, i.e., deletion of the *Ssp*I-to-*Sca*I section and insertion of a termination codon toward the 5′ end of the coding sequence (*Eco*57 site). Initiation of RNA synthesis at the *leu-500* promoter was analyzed as before, and the results are shown in Figure 4. In contrast to the situation in which *bla* was initiated at its normal promoter, with the *tac* promoter present the effect of *bla* deletion and translational termination was less significant. Initiation at the *leu-500* promoter was reduced relative to the construct with the unmodified *bla* gene, to 55 and 73% for the deletion and termination, respectively. However, *leu-500* expression remained 2-fold higher relative to its expression in the plasmid carrying the normal *bla* gene and promoter (i.e., pLEU500Tc), even when *bla* translation was terminated almost at the N-terminus of the protein. This suggests that the high level of leftward transcription when the *tac* promoter is present obviates the requirement for translation of *bla* for the activation of the *leu-500* promoter observed in its absence. It is possible that the additional mass of ribosomes attached to the *bla* mRNA is required for significant effects upon local superhelicity when the gene is initiated from a weak promoter but that this becomes less important as the number of elongating RNA polymerases is increased due to a stronger promoter.

Alterations to tetA Modulate the Activity of the leu-500 Promoter in pLEU500P$_{tac}$bla. Given that initiation of *leu-500* RNA synthesis was significantly increased when the *leu-500* promoter was adjacent to the inserted *tac* promoter, we wondered if the *tetA* gene might become dispensable in this plasmid. We therefore made a number of alterations to the *tetA* gene in pLEU500P$_{tac}$*bla* (Figure 5). Removal of the *tetA* promoter by deletion of the 166-bp *Eco*RV fragment reduced the level of initiation at the *leu-500* promoter to the experimental background. Introduction of termination codons

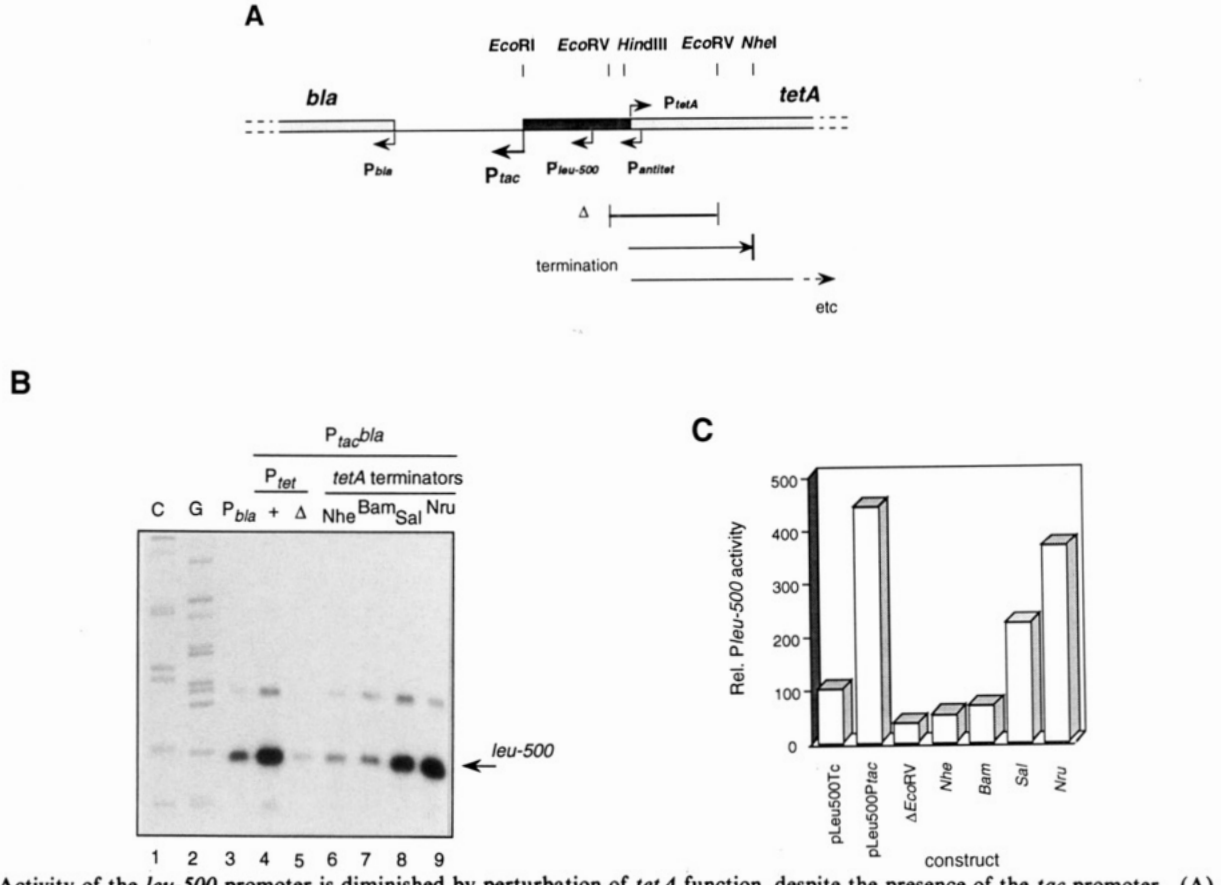

FIGURE 5: Activity of the *leu-500* promoter is diminished by perturbation of *tetA* function, despite the presence of the *tac* promoter. (A) Map of the relevant part of pLEU500P$_{tac}$*bla*, showing the modifications made to the plasmid in and around the *tetA* gene. A deletion was made by excision of the region between the *Eco*RV sites, removing both the *tetA* and *antitet* promoters. Translational termination codons were introduced into the *Nhe*I, *Bam*HI, *Sal*I, and *Nru*I sites. (B) Autoradiograph of sequencing gel showing results of reverse transcription of cellular RNA extracted from CH582. Transcription initiating at the *leu-500* promoter generates the band indicated by the arrow shown on the right. Tracks 1 and 2 contain C and G dideoxy sequencing markers using the internal *S. typhimurium* sequence primer. Track 3 contains cDNA generated from cellular RNA extracted from cells containing pLEU500Tc; the plasmid lacking the *tac* promoter, and track 4 contains the analogous experiment using pLEU500P$_{tac}$*bla* (confirming the large increase in *leu-500* promoter activity with the introduction of the *tac* promoter at the *Eco*RI site). Track 5 shows the effect of deleting the *tetA* promoter from pLEU500P$_{tac}$*bla*—note the large reduction in activity of the *leu-500* promoter. Tracks 6–9 show the effects of *tetA* translation termination on the activity of the *leu-500* promoter (terminators inserted into the *Nhe*I, *Bam*HI, *Sal*I, and *Nru*I sites, respectively). (C) The data of (B) were quantified by phosphorimaging and are presented as a histogram showing relative degrees of initiation at the *leu-500* promoter in the different constructs. Note again the large increase in the activity of the *leu-500* promoter with the introduction of the *tac* promoter. However, even with the *tac* promoter present, removal of the *tetA* promoter results in a considerable reduction in activity of the *leu-500* promoter (Δ*Eco*RV). The effect of termination of *tetA* translation is clear (indicated by restriction sites), with the activity of the *leu-500* promoter decreasing as the length of the TetA polypeptide synthesized is reduced.

at different positions in the coding sequence of *tetA* also reduced the activity of the *leu-500* promoter. We observed a progressive reduction in activity of the *leu-500* promoter as the length of TetA peptide synthesised was reduced. The effect of both promoter deletion and termination of translation was closely similar to the equivalent modifications made to pLEU500Tc (Chen et al., 1992), showing the dominant effect of *tetA* expression even in the presence of the strong *tac* promoter.

Effect of Spacing between the leu-500 and tetA or bla Promoters. Identification of the region between the *tetA* and *bla* genes as a discrete topological domain raises the question of the possible influence of the size of this segment of the plasmid. We were interested in knowing if the spacing between the *tetA* and *leu-500* promoters could be varied without affecting the activation of the latter or if initiation of transcription at *leu-500* would impose restraints on the spacing between the two. We therefore cloned additional DNA segments between the *tetA* and *leu-500* promoters of pLEU500Tc and examined the level of RNA synthesis initiated from the *leu-500* promoter in CH582 as before. The construction of these plasmids was carried out in two stages (see Materials and Methods for details). A 44-bp sequence was initially introduced into the *Hin*dIII site of pLEU500Tc at the *tetA* promoter to create the plasmid pLEU500 *Xba*. The insertion of this sequence recreated the sequences upstream of the *tetA* promoter and generated a new *Xba*I site 5′ to these sequences. Additional sequences were then cloned into this *Xba*I site to create additional spacer sequences between the *tetA* and *leu-500* promoters. The results are shown in Figure 6B. It is clear that the introduction of spacer DNA between the two promoters has a significant effect on the activity of the *leu-500* promoter; the level of *leu-500* promoter activity depends on the spacing from the *tetA* promoter, falling to a minimum level for spacings greater than 186 bp.

As a result of these observations, we asked whether or not the spacing between *leu-500* and *bla* promoters might affect initiation of transcription of the former. We therefore cloned some of the same spacer fragments of DNA into the *Eco*RI site of pLEU500Tc and examined the effect on the activity of the *leu-500* promoter (Figure 6C). There was a marked reduction in promoter activity at the *leu-500* promoter when an additional 754 or 1497 bp were introduced between it and the *leu-500* promoter, comparable with the reduction in activity

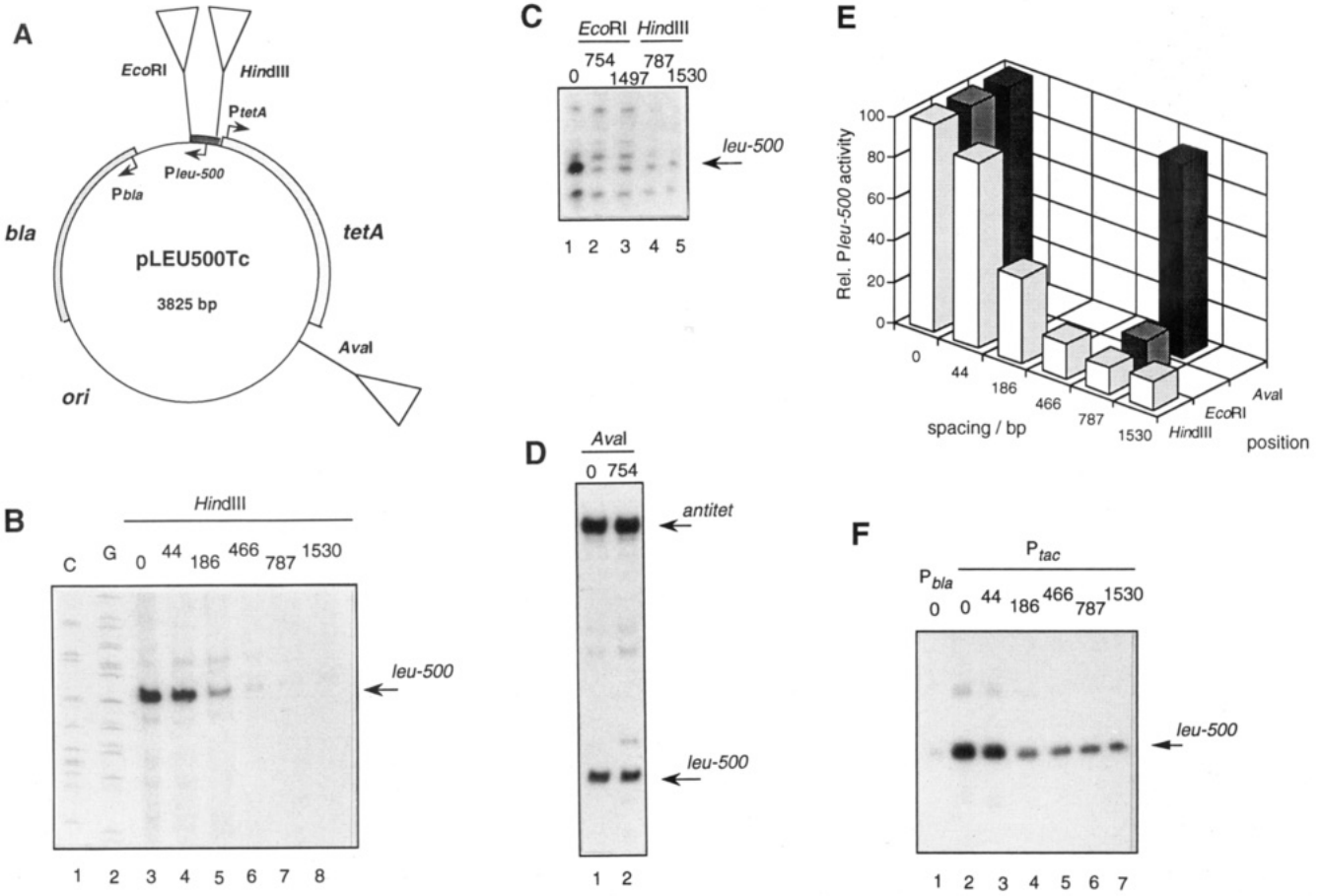

FIGURE 6: Dependence of the activity of the *leu-500* promoter on its spacing from other promoters. (A) Circular map of pLEU500Tc showing the location of insertion of the spacer DNA fragments. (B) Effect of introducing spacer fragments between the *tetA* and *leu-500* promoters. Autoradiograph of sequencing gel showing results of reverse transcription of cellular RNA extracted from CH582. Transcription initiating at the *leu-500* promoter generates the band indicated by the arrow shown on the right. Tracks 1 and 2 contain C and G dideoxy sequencing markers. Tracks 3–8 contain cDNA generated from cellular RNA extracted from cells containing pLEU500Tc and its derivatives. Track 3 was derived from pLEU500Tc without additional spacer fragments, and Track 4 from pLEU500 *Xba* which contains an additional 44 bp. The remaining tracks were derived from insertions into the *Xba*I site of pLEU500 *Xba*, with the total size of the insertion (with respect to pLEU500Tc) shown above the tracks. The reduced activity of the *leu-500* promoter as the spacing increases is clear. (C) Effect of introducing spacer fragments between the *bla* and *leu-500* promoters. Autoradiograph of sequencing gel showing results of reverse transcription of cellular RNA extracted from CH582. Track 1 was derived from pLEU500Tc without additional spacer fragments. Tracks 2 and 3 were derived by insertion of spacer fragments into the *Eco*RI site, and tracks 4 and 5 show the result of the equivalent insertions into the modified *Hin*dIII site as in (B). (D) Effect of introducing spacer fragments outside the *bla*-to-*tetA* region of the plasmid. Autoradiograph of sequencing gel showing results of reverse transcription of cellular RNA extracted from CH582. Track 1 was derived from pLEU500Tc without additional spacer fragments, while track 2 was derived by insertion of a 754-bp spacer fragment into the *Ava*I site. (E) The data of (B)–(D) were quantified by phosphorimaging and are presented as a histogram showing relative degrees of initiation at the *leu-500* promoter in the different constructs. Note the pronounced reduction in activity of the *leu-500* promoter when spacer fragments were introduced either between the *tetA* and *leu-500* promoters or between the *bla* and *leu-500* promoters. However, insertion of the 754-bp fragment 3′ to the *tetA* gene (*Ava*I site) had minimal effect on the activity of the *leu-500* promoter. (F) Effect of introducing spacer fragments between the *tetA* and *leu-500* promoters in the presence of the *tac* promoter at the *Eco*RI site. The constructs are the exact equivalent of those in (B), except that the parent plasmid is PLEU500P$_{tac}$*bla*. Autoradiograph of sequencing gel showing results of reverse transcription of cellular RNA extracted from CH582. Track 1 contains cDNA generated from cellular RNA extracted from cells containing pLEU500Tc, the plasmid lacking the *tac* promoter, and track 2 contains the analogous experiment using pLEU500P$_{tac}$*bla* (again demonstrating the large increase in *leu-500* promoter activity with the introduction of the *tac* promoter). Track 3 was derived from pLEU500P$_{tac}$*bla Xba* which contained an additional 44 bp, and the remaining tracks were derived from insertions into the *Xba*I site of pLEU500P$_{tac}$*bla Xba*, with the total size of the insertion (with respect to pLEU500P$_{tac}$-*bla*) shown above the tracks. Note that even with the longest insertions into pLEU500P$_{tac}$*bla*, the activity of the *leu-500* promoter was still significantly greater than that in pLEU500Tc.

when the same fragments were interposed between the *leu-500* and *tetA* promoters.

Both of the above spacing experiments have the effect of increasing the overall length of the domain enclosed by the *bla* and *tetA* promoters. We therefore asked whether introduction of spacer DNA into a different region of the circular plasmid would affect the activity of the *leu-500* promoter, and we cloned the same 754-bp fragment into the *Ava*I site 3′ to the *tetA* gene. Once again the expression of the *leu-500* promoter was measured in CH582 (Δ*topA*), and the results are presented in Figure 6D. In contrast to the experiments above, introducing additional DNA sequences into the plasmid in a location outside the *bla*-to-*tetA* domain had a minimal effect on the initiation of RNA synthesis at the *leu-500* promoter; initiation at the *leu-500* promoter was reduced to 93%, compared to <15% when the same fragment was introduced at locations within the *bla*-to-*tetA* domain.

The marked effect of introducing additional DNA sequences into the region between the *bla* and *tetA* promoters suggests that the increase in the overall size of the domain might dilute negative supercoiling arising from transcriptional activity. The additional sequences might therefore be better tolerated by the *leu-500* promoter in pLEU500P$_{tac}$*bla*, due to the effect of the stronger *tac* promoter noted above. We examined the

effect on RNA initiation at the *leu-500* promoter when we introduced the same series of spacer fragments between the *tetA* and *leu-500* promoters of pLEU500P$_{tac}$*bla* (Figure 6F). While there remained a clear dependence on the distance between these two promoters, the overall level of activity of the *leu-500* promoter was significantly higher for all plasmids in the series. Thus even with an additional 1530 bp placed between the *tetA* and *leu-500* promoters, *leu-500* promoter activity still remained higher than that measured in pLEU500Tc, where there was no spacer DNA.

DISCUSSION

The conclusion emerging from these results is that the activation of the *leu-500* promoter on a plasmid in a Δ*topA* strain is a function of its position in the domain located between the *tetA* and *bla* genes. We earlier demonstrated the importance of transcription and translation of the *tetA* gene (Chen et al., 1992), and the importance of the N-terminal peptide of TetA is consistent with a requirement for membrane location. We have now shown that *bla* can also play a role in the activation of the *leu-500* promoter. Either partial deletion of *bla* or premature termination of β-lactamase translation leads to a significant loss in transcriptional initiation at the *leu-500* promoter, and elevated leftward transcription due to an inserted *tac* promoter gives rise to a large increase in *leu-500* promoter activity. This effect is not peculiar to *bla*, as we have replaced it by a partially truncated *tetA* gene and observed a 2-fold increase in *leu-500* promoter activity (data not shown). Transcription appears to be the major activity required for the leftward gene; translation appears to be less important, especially when transcription is boosted by the insertion of a stronger promoter. Despite the importance of the leftward promoter, the *tetA* promoter is indispensable in the activation of the *leu-500* promoter, even when the *tac* promoter is present. For example, when the *tetA* promoter was excised from pLEU500P$_{tac}$*bla*, the activity of the *leu-500* promoter fell virtually to background levels.

The twin supercoiled-domain model of Liu and Wang (1987) provides a framework on which to rationalize these observations. The results are consistent with the existence of a topological domain between the *tetA* and *bla* genes of pLEU500Tc, in which transcription-induced negative supercoiling leads to an elevated level of negative superhelix density that cannot be efficiently relaxed in a *topA* strain. The increased supercoiling would be responsible for the activation of the *leu-500* promoter according to this viewpoint. Two circumstances are essential for the activation of the *leu-500* promoter. First, a *topA* background is required because topoisomerase I would relax negative supercoiling in the domain in a *topA*$^+$ strain. Second, transcription, translation, and membrane insertion of *tetA* play indispensable roles, generating the anchor that prevents rotation of the DNA from providing an alternative method of relaxation of negative supercoiling by superhelical diffusion. TetA is well suited to this role. The metal–tetracycline/proton antiporter is located in the inner membrane of the cell with 12 transmembrane segments (Allard & Bertrand, 1992; Eckert & Beck, 1989), and membrane insertion is coupled to transcription and translation. Genes for exported proteins such as *phoA* cannot substitute for *tetA* (data not shown). The particular role of *tetA* in the oversupercoiling of plasmids in *topA* strains has been demonstrated (Lodge et al., 1989; Pruss & Drlica, 1986), but Lynch and Wang (1993) have shown that *tetA* can be replaced by genes such as *lacY* or *tolC* that are similarly subject to coupled transcription, translation, and insertion.

Although β-lactamase is a periplasmic enzyme, its export is not coupled to synthesis (Koshland & Botstein, 1982), and therefore its role as a second anchor seems to be excluded. This is consistent with our results which show a relatively minor requirement for translation of the *bla* gene in the activation of the *leu-500* promoter. If a second anchor point is required, it must be another feature of the plasmid, such as the replication origin. Nevertheless, our results indicate that transcription that is divergent with respect to *tetA* is important for the activation of the *leu-500* promoter, even without membrane contact. Thus simple transcription on the circular plasmid can have important consequences for the topological coupling of promoters. This observation appears to conflict with earlier studies of plasmid supercoiling in Δ*topA* strains, where no effect of *bla* expression has been found (Lodge et al., 1989; Pruss & Drlica, 1986). However, these investigations were made by measurement of the linking differences of plasmid DNA extracted from cells, whereas the level of *in vivo* activity of the *leu-500* promoter may reflect effects that are restricted to the local domain and that may become masked in a global measurement of linkage.

The size of the domain between the two divergent transcription units is important. We observed marked reduction in the initiation of RNA synthesis at the *leu-500* promoter when DNA fragments of 180 bp or longer were placed anywhere within the *bla*-to-*tetA* domain (but not outside it). The simplest explanation for these results would be that the transcription-induced supercoiling in the domain becomes "diluted" by the increased size of the region between *bla* and *tetA* genes; addition of 186 bp to the DNA in this domain increases its size by a factor of 1.5. The steady-state superhelix density in this region should be a function of the relative rates of induction by transcription and relaxation and the length of DNA between the genes. We see that the proposed consequence of supercoiling in the domain (i.e., activity of the *leu-500* promoter) responds consistently to increased or reduced transcription, increased domain size, and combinations of these changes. Thus, boosting leftward transcription by the relatively strong *tac* promoter results in a significant level of activity of the *leu-500* promoter even when the domain size is increased fivefold.

In conclusion, the activation of the *leu-500* promoter on a plasmid is dependent on three factors, viz., the absence of topoisomerase I activity, the existence of a topological domain lying between actively transcribed divergent promoters, and the anchorage of one transcription unit to the lipid bilayer membrane by coupled transcription, translation, and insertion. The *leu-500* promoter system provides an example of the complex interactions that are possible between two or more promoters, leading to the modulation of the function of one promoter by the activity of another. The medium of this interaction appears to be the topology of the DNA template. All the available data are consistent with the notion that the *leu-500* promoter becomes activated by negative supercoiling arising from transcription of the divergent genes, and we have direct physical evidence for elevated negative supercoiling upstream of the *tetA* gene in Δ*topA* strains (R. Bowater, D. Chen, and D. M. J. Lilley, manuscript in preparation). We have previously termed this property the topological coupling of promoters and suggested that it could be general. Such mechanisms could result in promoter cooperativity or anticooperativity, as well as the coupling of transcription to other events such as DNA replication (Baker & Kornberg, 1988) or recombination (Dröge, 1993). It might be argued that the apparent requirement for a *topA* background would limit the

relevance of the phenomenon; however, we have recently obtained evidence of significant oversupercoiling upstream of the *tetA* gene in *top*$^+$ *Escherichia coli* (R. Bowater, D. Chen, and D. M. J. Lilley, manuscript in preparation). Thus there is no fundamental impediment to a more general biological exploitation of these effects.

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