Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8784–8788, September 1992
Biochemistry

Activity of a plasmid-borne *leu-500* promoter depends on the transcription and translation of an adjacent gene

(DNA supercoiling/twin supercoiled domains/*leu-500* mutation/topological coupling/topoisomerase)

DONGRONG CHEN, RICHARD BOWATER, CHARLES J. DORMAN, AND DAVID M. J. LILLEY*

Department of Biochemistry, The University, Dundee DD1 4HN, United Kingdom

Communicated by James C. Wang, June 23, 1992

ABSTRACT ***leu-500* is a chromosomal promoter mutation in *Salmonella typhimurium* that normally causes the promoter to be inactive in the initiation of RNA synthesis. But in a strain that has mutations in *topA*, the gene encoding DNA topoisomerase I, the mutant promoter becomes active. We show that the *leu-500* promoter can function on a plasmid when it is adjacent to the tetracycline-resistance gene *tetA*. Activation of the *leu-500* promoter requires that the *tetA* gene is transcribed and translated and that the host cell is *topA*. We propose that the A → G mutation in the −10 region of the *leu-500* promoter is compensated by local negative supercoiling arising from transcription of the *tetA* gene, which may reach elevated levels in a *topA* background, provided that diffusional dissipation is reduced due to anchoring of the TetA peptide in the membrane. This is a clear example of the modulation of the activity of a promoter by the activity of another promoter in cis, when they can be coupled through the topology of the template.**

Transcription and DNA supercoiling are closely interlinked through the topology of the DNA template at a number of levels. This arises in part because local strand separation is required for transcription to take place (1). Initiation of transcription requires alterations to the template topology that are coupled to DNA supercoiling (2), and examples of promoters that are sensitive to DNA supercoiling have been described (reviewed in ref. 3). However, as we show here, this coupling may be quite complex.

The promoter of the *Salmonella typhimurium* leucine biosynthetic operon is interesting from this point of view. A mutant (*leu-500*) was isolated that resulted in leucine auxotrophy (4), which was subsequently discovered to be an A → G transition mutation in the −10 sequence of the promoter (5). A suppressor mutation (*supX*) had been isolated that restored leucine prototrophy (6); *supX* was identified as the structural gene for DNA topoisomerase I, *topA* (7, 8). This suggested a simple mechanism for the suppression of the *leu-500* mutation (9, 10). Replacement of the A·T by a G·C base pair in the −10 region should make the formation of the open complex more energetically expensive, but the extra energy might be provided by negative supercoiling. A *topA* strain would lack the main enzyme involved in the relaxation of negative supercoiling, and thus the mean level of supercoiling should rise; this has been confirmed experimentally (11, 12). This increase might provide the additional free energy required for the *leu-500* promoter to function. It should be noted that any null mutation in *topA* results in Leu$^+$ cells.

Inconsistencies with this simple model emerged from our studies of the genetic control of DNA supercoiling in *S. typhimurium* (11). We observed a perfect correlation between leucine prototrophy and the presence of *topA* mutation, even when compensatory mutations *reduced* the level of negative supercoiling. Thus the correlation was not with the measured levels of plasmid supercoiling but with the presence or absence of topoisomerase I, suggesting a local role for the enzyme. A second problem emerged in that the suppression was apparently restricted to the chromosomal promoter; we could not activate the *leu-500* promoter on a plasmid (13).

Liu and Wang (14–18) have proposed a model that shows how supercoiling can be considered as a local phenomenon. In this view RNA polymerase experiences hindrance to free rotation, and its forward passage along the DNA helix generates a domain of positive supercoiling ahead, and one of negative supercoiling behind, the elongation complex. The steady-state levels of supercoiling in these domains will reflect a balance between their rate of induction and their relaxation. Supercoils may be relaxed either by diffusion along the DNA or by the action of enzymes. In prokaryotes positive and negative supercoils are relaxed predominantly by DNA gyrase and DNA topoisomerase I, respectively. The twin supercoiled-domain model is consistent with observations of DNA supercoiling following the inhibition of topoisomerases (19, 20).

To explain the *leu-500* data we postulate the existence of a second, divergent promoter (21) (Fig. 1). An open reading frame is present upstream of, and divergent to, the *leu* genes of *S. typhimurium* and *Escherichia coli* (22). The negative supercoiling arising from the transcription of this promoter will normally be relaxed by topoisomerase I and should attain a higher steady-state level in a *topA* strain—this local increase could activate the *leu-500* promoter. Because the domain of negative supercoiling can only be relaxed by topoisomerase I, this explains why the promoter is only activated in a *topA* strain and is not affected by *gyr* mutations. The model also provides an explanation for the failure of *topA* mutations to activate *leu-500* on a plasmid, because in this case a second mechanism of relaxation becomes dominant. On a circular DNA molecule, positive and negative domains of supercoiling arising from a transcription complex can cancel by diffusion around the molecule and local rotation of the DNA (Fig. 1). Thus the domains of supercoiling may never reach the steady-state level required to activate *leu-500*, even in the absence of topoisomerase I activity.

A test of this model might be possible if we could provide a barrier to the diffusion of the superhelical tension around the plasmid. Pruss and Drlica (19) showed that *topA*-dependent supercoiling of pBR322 required transcription of the tetracycline-resistance gene *tetA*, and Berg and coworkers (23) showed that export of the TetA protein into the cell membrane anchors the plasmid DNA. Such membrane anchoring might provide the barrier to superhelical diffusion required for the activation of *leu-500*. We have therefore constructed a plasmid in which the *leu-500* promoter was placed divergent to the *tetA* gene, whereupon we found that

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*To whom reprint requests should be addressed.

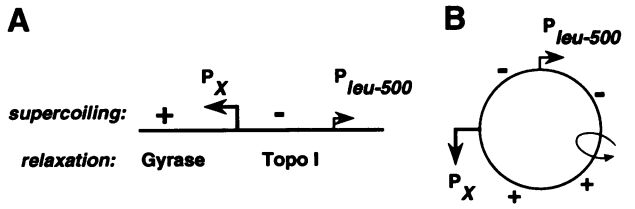

FIG. 1. Model for suppression of the *leu-500* promoter mutation by mutations in *topA*. (*A*) The model postulates the existence of a cryptic promoter (P_X) divergent to the *leu-500* promoter, transcription from which generates domains of positive and negative supercoiling according to the twin supercoiled-domain model of Liu and Wang (14). In a *topA* mutant the local steady-state level of negative supercoiling behind P_X is increased, and this may facilitate the activation of the mutant *leu-500* promoter. Topo I, topoisomerase I. (*B*) On a circular plasmid positive and negative domains of supercoiling arising from any promoter may diffuse around the circle and cancel by a rotation of the DNA.

the *leu-500* promoter may now be activated on the plasmid by *topA* mutation. The function of the *leu-500* promoter required transcription and translation of the *tetA* gene, in a manner fully consistent with the proposed mechanism of activation.

MATERIALS AND METHODS

Growth of Bacterial Strains. All bacterial strains were derivatives of *S. typhimurium* LT2 as described in Richardson *et al.* (11). CH582 is Δ*topA2762 leu-500 ara-9*. Bacteria were cultured at 37°C in liquid media or on 1.2% agar plates. All strains were grown in Luria broth (LB) to repress transcription from the chromosomal leucine promoter. Media were supplemented with antibiotics as required—either ampicillin at 50 μg/ml or tetracycline at 10 μg/ml. Plasmids were transformed into cells by the calcium chloride method (24).

Plasmid Construction. *Eco*RI–*Hin*dIII fragments of the pED101-based plasmids (13) containing the *leu-500* or the wild-type leucine promoters (*leuP*) were cloned into the *Eco*RI–*Hin*dIII site of pAT153 to give pLEU500Tc and pLEUPTc. Although the original −35 and −10 elements of the tetracycline promoter were altered by this procedure, these plasmids retained tetracycline resistance.

Disruption of the tetracycline promoter. This was achieved by two methods. Small promoter deletions were obtained by cleavage of pLEU500Tc and pLEUPTc at the *Hin*dIII site and digesting the termini by incubation with 6 units of S1 nuclease in 50 mM sodium acetate, pH 4.5/50 mM NaCl/1 mM $ZnCl_2$/5% glycerol on ice for 4 min. The blunt-end fragments were ligated with T4 DNA ligase. The second procedure involved digestion with *Eco*RV, isolation of the large fragment by electrophoresis, and religation, generating plasmids that had lost the *tet* and *antitet* promoters (see Fig. 4).

Plasmids containing translation terminators within tetA. The following complementary oligonucleotides were ligated into the plasmids pLEU500Tc and pLEUPTc linearized by the appropriate restriction enzyme sequences: *Nhe* I, CTAGGCTAGGCTAG and CTAGCTAGCCTAGC; *Bam*HI, GATCTAGCTAGCTAG and GATCCTAGCTAGCTA; *Sal* I, TCGACTAGCTAGCTAG; *Nru* I CTAGCTAGCTAG. Plasmids were transformed into *S. typhimurium* LT2 and CH582, and DNA was sequenced by primer extension (25).

RNA Extraction and 5′ End Analysis. RNA was prepared from freshly inoculated cultures at midlogarithmic phase. Two hundred microliters of cultures plus an equal volume of 20 mM sodium acetate, pH 5.2/2% SDS/0.3 M sucrose were placed in a boiling water bath for 1 min. This was phenol extracted and the nucleic acids were precipitated with ethanol. After 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 8.0/50 mM KCl and rapidly cooled. Twenty-five units RNasin (0.5 μl) was 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/1.3 mM dNTPs containing 6 units of avian myeloblastosis virus reverse transcriptase and incubation at 42°C for at least 1.5 hr. Transcripts were electrophoresed in 6% polyacrylamide in 90 mM Tris borate, pH 8.3/10 mM EDTA (TBE buffer) containing 7 M urea next to sequence markers generated by dideoxy sequence reactions (25) using the same primer. For samples from the *leu-500* plasmids all of the solution was loaded on the gel; for those from *leuP* plasmids, only 1/10th of the volume was loaded. 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, Sunnyvale, CA). Quantitation was performed upon the phosphorimage.

Analysis of Linking Number of Extracted Plasmid DNA. Cells were grown in LB to midlogarithmic phase as above, harvested, and lysed (26). Purified DNA was electrophoresed in 1% agarose in TBE containing chloroquine. Gels were stained in ethidium bromide at 1 μg/ml and photographed under UV illumination.

RESULTS

The *leu-500* Promoter Functions on a Plasmid Carrying the Tetracycline-Resistance Gene. We have previously noted (13) the failure of *topA* to suppress the *leu-500* promoter mutation on a plasmid (confirmed by primer extension; data not shown), which we explain by postulating superhelical diffusion. From the experiments of Lodge *et al.* (23) we suspected that expression of the tetracycline-resistance gene might provide a topological barrier on a plasmid that might allow a sufficient buildup of local negative supercoiling to permit the *leu-500* promoter to function on a circular molecule. We therefore constructed the plasmid shown in Fig. 2 by excision of the 199-base-pair (bp) *Eco*RI to *Hin*dIII fragment of *S. typhimurium* DNA containing either the *leu-500* or *leu-P* promoter and inserting it into the corresponding sites of pAT153. The resulting plasmids (pLEU500Tc and pLEUPTc) contained the *leu* and *tet* promoters arranged divergently. The *antitet* promoter (the promoter of the absent tetracycline repressor gene *tetR*) was also present in the same orientation as the *leu* promoter. Initiation of RNA synthesis was analyzed using cDNA synthesis by avian myeloblastosis virus reverse transcriptase, with a primer corresponding to vector sequence beyond the *S. typhimurium* DNA, which was therefore specific for RNA synthesized from the plasmid rather than the chromosome.

The results are shown in Fig. 3. In contrast to the results with the pED101-based plasmids, a band of cDNA corresponding to initiation at the *leu-500* promoter is now evident, although the extent of RNA synthesis is considerably lower than that arising from the *leu-P* promoter.

Initiation of Transcription at *leu-500* Depends on the Presence of *topA*. Reverse transcriptase analysis of transcripts from pLEU500Tc and pLEUPTc was performed for RNA extracted from *topA*$^+$ and Δ*topA S. typhimurium* strains (Fig. 3 *A* and *B*). It can be seen that in the *topA*$^+$ strain there was no initiation at the *leu-500* promoter, and thus the function of the mutant promoter was totally dependent on the *topA* background. This was not true of the *leu-P* promoter, which was functional irrespective of the presence or absence of *topA*.

We also examined the linking number of pLEU500Tc extracted from exponential LT2 (*top*$^+$) and CH582 (Δ*topA*) cells by means of electrophoresis in an agarose gel with

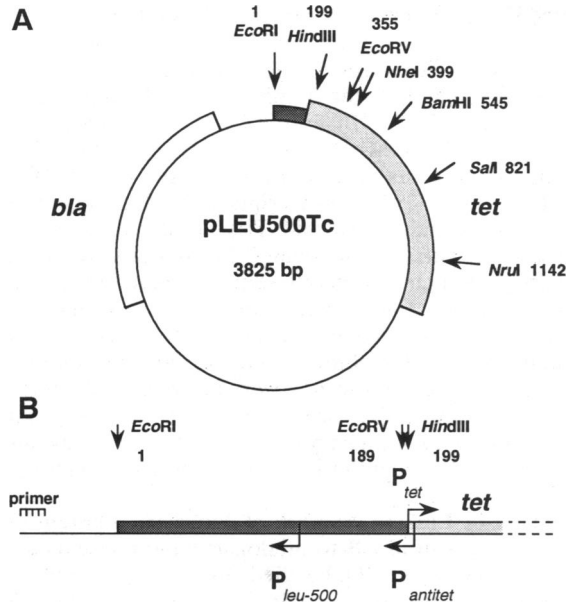

FIG. 2. Construction of a plasmid containing the *leu-500* promoter oriented divergently to the tetracycline-resistance gene. (*A*) Circular map of pLEU500Tc. The *S. typhimurium* fragment containing the *leu-500* promoter (stippled, dark) was cloned immediately adjacent to the *tetA* gene (stippled, light). A corresponding plasmid, pLEUPTc, was constructed in which the wild-type leucine sequence replaced the mutant fragment. Restriction sites used in these experiments are shown. (*B*) Linear map of the region around the *leu* fragment, showing the position and orientation of the *leu-500*, *tet*, and *antitet* promoters. Note the location of the primer used to study initiation of transcription in these studies; this lies outside the *S. typhimurium* DNA, in vector sequences, and therefore detects transcription arising only from the plasmid-borne promoter.

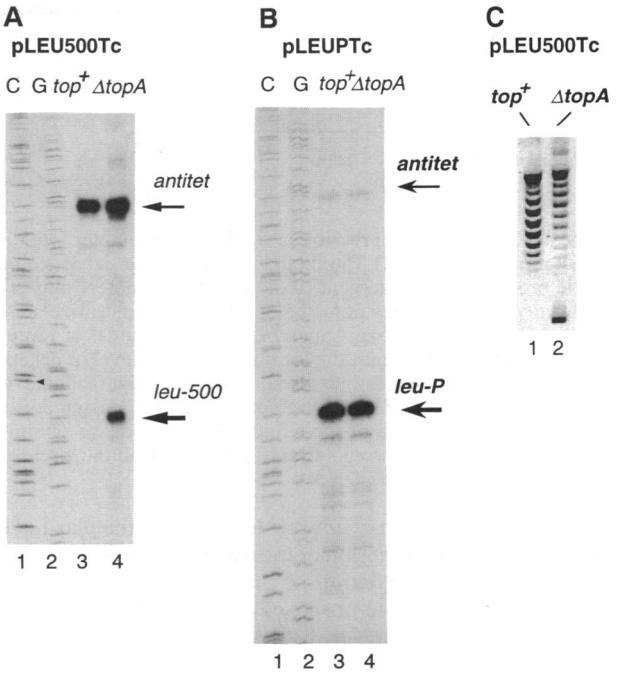

FIG. 3. Activation of the *leu-500* promoter on a plasmid containing the tetracycline-resistance gene. (*A*) Initiation of RNA synthesis in pLEU500Tc. Run-off transcripts were made from RNA extracted from *top*$^+$ (track 3) and Δ*topA* (track 4) *S. typhimurium* strains. Transcription initiating at the *leu-500* promoter generates the band indicated by the lower arrow shown on the right. Transcription initiating at the *antitet* promoter generates the band indicated by the upper arrow. The *leu-500* mutation is indicated on the C sequence lane (track 1). Note that the *leu-500* promoter is only functional in the Δ*topA* background. (*B*) Initiation of RNA synthesis in pLEUPTc. Run-off transcripts were made from RNA extracted from *top*$^+$ (track 3) and Δ*topA* (track 4) strains. Transcription initiating at the *leu-P* and *antitet* promoters generates the bands indicated by the lower and upper arrows, respectively. Note that the *leu-P* promoter is functional in both backgrounds, in contrast to the *leu-500* promoter. (*C*) Analysis of plasmid DNA supercoiling. Supercoiled pLEU500Tc DNA was isolated from *top*$^+$ (track 1) and Δ*topA* (track 2) strains in exponential growth. DNA was analyzed by electrophoresis in agarose in a buffer containing chloroquine at 3 μg/ml; other intercalator concentrations were used in additional experiments (data not shown), confirming that the topoisomers were all negatively supercoiled. DNA isolated from the Δ*topA* strain shows a bimodal topoisomer distribution with a fraction of highly negatively supercoiled DNA.

chloroquine contained in the buffer (Fig. 3*C*). In contrast to DNA isolated from the *top*$^+$ cells, pLEU500Tc extracted from the Δ*topA* cells exhibited a bimodal topoisomer distribution, and a fraction of the DNA was strongly oversupercoiled. No such oversupercoiling was observed in DNA of plasmids that lacked the *tetA* gene.

Initiation of Transcription at *leu-500* Is Severely Reduced by Deletion of the *tet* Promoter. We generated two additional plasmids from pLEU500Tc that were deleted in and around the promoter of the *tetA* gene (Fig. 4). The smaller deletion (pLEU500TcΔ10) was produced by cleavage with *Hin*dIII and treatment with S1 nuclease to remove 10 bp from the promoter. The larger deletion (pLEU500TcΔ166) was generated by excision of a 166-bp *Eco*RV fragment from the promoter and the 5′ end of the *tetA* gene. The former deletion leaves the *antitet* promoter intact, whereas the larger deletion removes it. The results (Fig. 4) show that transcription in the Δ*topA* background from the *leu-500* promoter was severely reduced by either deletion. Equivalent deletions in pLEUPTc had almost no effect on initiation at the *leu-P* promoter (data not shown). It should be stressed that neither deletion affects the *leu-500* promoter sequences directly (at least 70 bp upstream of the *leu* startsite remain unaltered). Thus the suppression of the *leu-500* mutation in the Δ*topA* strain seems to be a result of transcription of the *tetA* gene.

Initiation of Transcription at *leu-500* Requires a Minimum Length of Translated TetA Product. Inhibition of protein synthesis by chloramphenicol at 150 μg/ml virtually abolished initiation at *leu-500* (data not shown), whereas the *leu-P* promoter remained active. To examine the effect of translation of the *tetA* gene product on initiation at the *leu-500* and *leu-P* promoters more specifically, we introduced termination codons into the coding sequence at various positions along the gene by cloning oligonucleotides into single restriction sites. By this means the length of the translated product could be progressively reduced. These plasmids were transformed into Δ*topA S. typhimurium*, and initiation of RNA was analyzed as before. The effect of shortening the gene product on initiation at the *leu-500* promoter is very clear (Fig. 5); the more the protein was reduced in size, the lower the extent of initiation at *leu-500*. The data were quantified by phosphorimaging and are shown graphically in Fig. 5*C*. It is clear that the initiation of RNA synthesis at the *leu-500* promoter was strongly dependent on translation of the *tetA* gene product, and the level of initiation appears to be almost linearly dependent on the size of the protein synthesized beyond the initial 100 amino acids. The corresponding effects on the *leu-P* promoter were much smaller, but interestingly there appears to be some influence of TetA translation.

DISCUSSION

We have shown that RNA polymerase can initiate at the *leu-500* promoter carried on a circular plasmid in *S. typhi-*

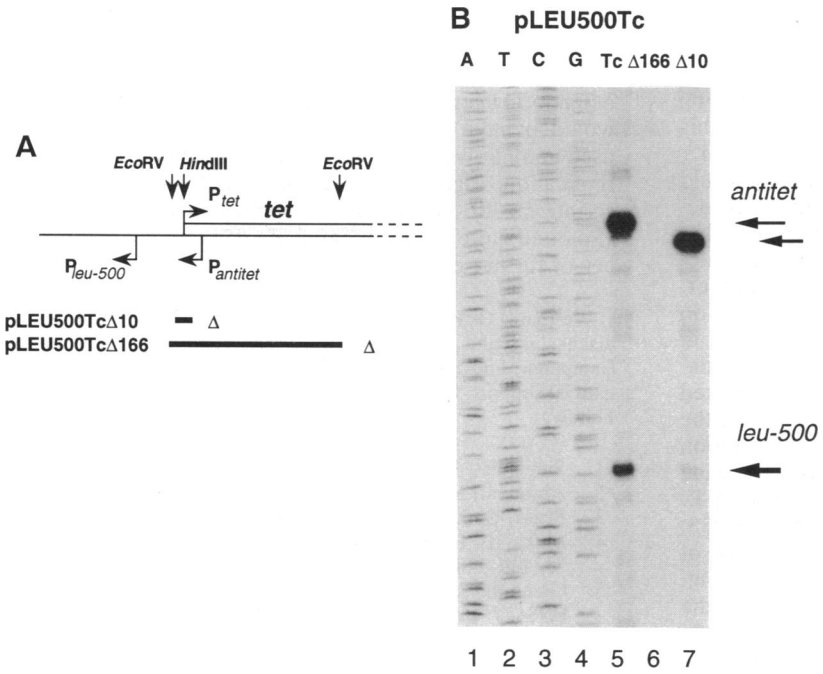

FIG. 4. *leu-500* promoter activity requires transcription of the tetracycline-resistance gene. (*A*) Deletions used in this study. pLEU500TC was deleted around the *tet* promoter either by *Hin*dIII cleavage and S1 nuclease trimming to generate a 10-bp deletion (pLEU500TcΔ10) or by removal of the 166-bp *Eco*RV fragment (pLEU500TcΔ166). The latter also removed the *antitet* promoter. (*B*) Initiation of RNA synthesis in promoter deletion plasmids in CH582 (Δ*topA*). Run-off transcripts were made from RNA extracted from unmodified pLEU500Tc (track 5) and the deletions pLEU500TcΔ10 (track 7) and pLEU500TcΔ166 (track 6). Transcription initiating at the *leu-500* and *antitet* promoters generated the bands indicated by the lower and upper arrows, respectively. Note that the *leu-500* promoter is not functional in either plasmid in which the *tetA* gene is not transcribed. Since the *antitet* promoter is deleted in pLEU500TcΔ166 (track 6) there is no band corresponding to initiation at this site.

murium. This requires that the cells are *topA* and that the plasmid is transcribing and translating the tetracycline-resistance gene. Thus the *topA*-dependent functioning of the *leu-500* promoter is linked to the expression of a neighboring gene.

These data are fully consistent with a model (21) (Fig. 1) in which the local supercoiling arising from the transcription of a neighboring divergent gene is elevated in a *topA* strain, thereby facilitating the function of the *leu-500* promoter. The *leu-500* mutation is in the −10 region of the promoter (5),

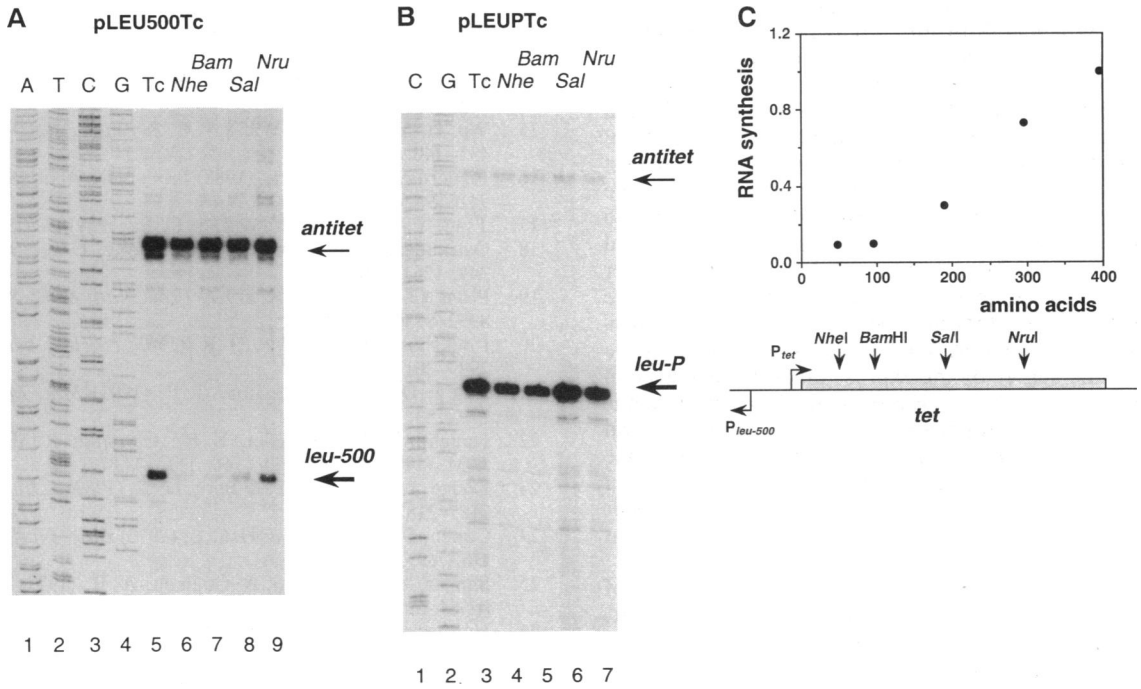

FIG. 5. *leu-500* promoter activity requires translation of the tetracycline-resistance gene: activity of plasmid-borne *leu* promoters in CH582 (Δ*topA*) in plasmids derived from pLEU500Tc and pLEUPTc in which termination codons have been introduced at various positions in the *tetA* gene. (*A*) Initiation of RNA synthesis from the *leu-500* promoter in plasmids derived from pLEU500Tc. Tracks 1–4, sequence markers; track 5, RNA initiation from the *leu-500* promoter in the unmodified plasmid; tracks 6–9, termination codons were introduced into the *Nhe* I, *Bam*HI, *Sal* I, and *Nru* I sites, respectively (see Fig. 2). Note the variation in the extent of initiation at the *leu-500* promoter with the positions of terminators within the *tetA* gene. (*B*) Initiation of RNA synthesis from the *leu-P* promoter in corresponding plasmids derived from pLEUPTc. Track 3, RNA initiation from the *leu-P* promoter in the unmodified plasmid; tracks 4–7, termination codons were introduced as in *A*. Note the smaller variation in the extent of initiation at the *leu-P* promoter with the positions of terminators within the *tetA* gene. (*C*) Relative extent of initiation of RNA synthesis at the *leu-500* promoter as a function of the length of translated product. The relative initiation at the *leu-500* promoter was quantified by phosphorimaging and is plotted as a function of the expected chain length of TetA (relative to the unmodified plasmid). There is very little initiation at the *leu-500* promoter for TetA lengths shorter than 100 amino acids, and the activity of the promoter increases approximately linearly thereafter.

which is initially opened by the polymerase in the isomerization event (1). Although the *leu* promoter is some way from being a classical Pribnow box sequence, it contains a TpA step that may well play a fundamental role in initiating the strand separation reaction. In the *leu-500* promoter this sequence is changed to TpG, which is known to be of higher stability (27). However, elevated negative supercoiling would help to overcome this increased stability of the −10 sequence. In the current experiments we have shown that initiation of RNA synthesis at the *leu-500* promoter is dependent on transcription from the divergent *tet* promoter. The critical difference between the plasmid pLEU500Tc, on which the *leu-500* promoter is functional, and plasmids in earlier studies in which it was not appears to be the translation of the *tetA* gene product TetA. This protein is exported through the cell membrane and therefore serves to anchor the plasmid and prevent free rotation of the DNA. Transcription of the *tetA* gene was shown to be associated with wide topoisomer profiles in plasmid DNA isolated from *topA E. coli* cells (19) and the formation of Z-DNA inside the cell (28, 29), and Berg and coworkers (23) showed that translation of the *tetA* gene product was essential for oversupercoiling. This is confirmed in this study and strongly suggests that the anchoring provides a barrier to superhelical diffusion in the plasmid such that local negative supercoiling arising from transcription may rise to the point at which the *leu-500* promoter can function.

RNA synthesis arising from the *leu-500* promoter of pLEU500Tc was considerably less than that of the wild-type *leu-P* promoter in the same location, yet our earlier studies of the expression of the chromosomal promoters indicated that the suppressed *leu-500* promoter was of comparable efficiency to the *leu-P* promoter (13). This may indicate that a degree of superhelical diffusion may persist in the plasmid, which may not be possible on the chromosome.

In addition to explaining the suppression of the *leu-500* mutation, these experiments also demonstrate the potential significance of local supercoiling arising from transcription, as first suggested by Liu and Wang (14). These results suggest that such effects might have important consequences for gene expression in the bacterial cell. However, there are two questions that may be raised before the potential biological significance can be assessed. (*i*) *leu-500* is a mutant promoter and therefore a rather special example. However, there are many other promoters that may be affected by DNA supercoiling, and we notice that *leu-P* expression appears to be affected to some degree by the expression of the *tetA* gene in our experiments (Fig. 5). (*ii*) Expression of *leu-500* depends on the absence of DNA topoisomerase I in the *topA* cells, and the effect is not operative in wild-type cells. The significance of this is hard to assess at present. It is possible that if the *tet* promoter were replaced by a stronger promoter, or other strong promoters were added to the system, the effects might be detectable even in *topA*$^+$ cells.

The fundamental principle inherent in the suppression of the *leu-500* promoter mutation is potentially very significant. This is the control of one promoter by the activity of a second promoter, when they are coupled through the topology of the DNA template. Such topological coupling between promoters might take a number of different forms. For example, if two promoters that are both stimulated by negative supercoiling are arranged divergently, then they will act cooperatively. If one is inhibited by negative supercoiling, it would be topologically repressed by the activity of the other but might be stimulated if located at the 3′ end of an active neighboring gene. Two such promoters arranged divergently would behave anticooperatively. Similar effects might be used in other ways. For example, helix opening of the *E. coli* replication origin *oriC* by DnaA is activated by local transcription (30). In principle, topological coupling could be exploited quite generally, and one can anticipate the discovery of further examples.

We thank C. F. Higgins, D. Stirling, and M.-J. Giraud-Panis for discussions and the Medical Research Council, Cancer Research Campaign, and Science and Engineering Research Council for financial support. C.J.D. is a Royal Society 1983 University Research Fellow.

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