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 diffusion dissipates 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

*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+) 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+ 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 ΔtopA2762 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. EcoRI–HindIII fragments of the pED101-based plasmids (13) containing the leu-500 or the wild-type leucine promoters (leuP) were cloned into the EcoRI–HindIII 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 HindIII 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 ZnCl2/5% glycerol on ice for 4 min. The blunt-end fragments were ligated with T4 DNA ligase. The second procedure involved digestion with EcoRV, 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, CTAG-GCTAGGCTAG and CTAGCTAGGCCTAG; BamHI, GATCTAGCTAG and GATCCTAGCTAG; Sal I, TCAGCTAGGCTAG; 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 μmol of the appropriate 32P-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 MgCl2/15 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 build up 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) EcoRI to HindIII fragment of S. typhimurium DNA containing either the leu-500 or leuP 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 leuP 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 ΔtopA 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 leuP 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 (ΔtopA) cells by means of electrophoresis in an agarose gel with
chloroquine contained in the buffer (Fig. 3C). In contrast to DNA isolated from the top+ cells, pLEU500Tc extracted from the ΔtopA cells exhibited a bimodal topoisomer distribution, and a fraction of the DNA was strongly supercoiled. No such supercoiling 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 HindIII and treatment with S1 nuclease to remove 10 bp from the promoter. The larger deletion (pLEU500TcΔ166) was generated by excision of a 166-bp EcoRV 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 ΔtopA 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 ΔtopA 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 ΔtopA 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. 5C. 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-
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. 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 HindIII cleavage and S1 nuclease trimming to generate a 10-bp deletion (pLEU500TcA10) or by removal of the 166-bp EcoRV fragment (pLEU500TcA166). The latter also removed the antitet promoter. (B) Initiation of RNA synthesis in promoter deletion plasmids in CH582 (AtopA). Run-off transcripts were made from RNA extracted from unmodified pLEU500Tc (track 5) and the deletions pLEU500TcA10 (track 7) and pLEU500TcA166 (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 pLEU500TcA166 (track 6) there is no band corresponding to initiation at this site.

Fig. 5. leu-500 promoter activity requires translation of the tetracycline-resistance gene: activity of plasmid-borne leu promoters in CH582 (AtopA) 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, BamHI, 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 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 (22) 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 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 DnA 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.