Activation of the leu-500 Promoter by a Reversed Polarity tetA Gene

RESPONSE TO GLOBAL PLASMID SUPERCOILING*

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The leu-500 promoter is inactivated by a mutation in the −10 region but can be activated in topA Escherichia coli and Salmonella strains. We have found that the tetA gene plays a vital role in the topA-dependent activation of a plasmid-borne leu-500 promoter. In previous studies, the leu-500 promoter and tetA gene have been arranged divergently. In this study we have reversed the polarity of the tetA gene, thus locating the leu-500 promoter at the 3′ end of tetA. Despite being formally located in the downstream region of tetA, the leu-500 promoter is equally well activated in a topA strain in this environment, even though it is 1.6 kilobase pairs away from the promoter of the reversed tetA gene. Activation of the leu-500 promoter depends on transcription and translation of tetA but is largely insensitive to the function of other transcription units on the plasmid. These results require a change in viewpoint of the role of tetA, from local to global supercoiling. We conclude that transcription of the tetA gene is the main generator of transcription-induced supercoiling that activates the leu-500 promoter. Unbalanced relaxation of this supercoiling leads to a net increase in the negative link-difference of the plasmid globally, and there is a linear correlation between the change in global plasmid topology and the activation of the leu-500 promoter. Thus the leu-500 promoter appears to respond to the negative supercoiling of the plasmid overall.

The activation of the leu-500 promoter provides a good illustration of the possible interrelationships between transcription and the topology of the DNA template in vivo. leu-500 is a leucine auxotroph of Salmonella typhimurium (1) that results from an A to G transition in the −10 region of the promoter of the leu biosynthetic operon (2). It was found that leucine prototrophy was restored in Salmonella bearing a supX mutation (3). The later demonstration that supX was identical to topA (4), the gene encoding DNA topoisomerase I, provided a strong indication of a functional link between transcription and topology. Thus the increase in negative supercoiling that should arise from the loss of the supercoiling-relaxation activity from the Salmonella cell (5) might be expected to assist in the function of the leu-500 promoter, coupling the additional free energy of negative supercoiling to the opening of the more refractory −10 region of the mutant promoter (6, 7).

More recent work in this laboratory has identified an additional level of complexity in this process. The demonstration of a direct requirement for a null topA background (8) led to the suggestion that the leu-500 promoter might be activated by variations in template supercoiling arising from transcriptional-induced supercoiling due to the transcription of a nearby gene (9, 10). According to the twin-supercoiled domain model of Liu and Wang (11), a rotationally hindered RNA polymerase in the elongation phase of transcription will tend to generate positive supercoiling ahead of its passage and negative supercoiling in its wake. These domains will be relaxed by DNA gyrase and topoisomerase I, respectively, in eubacteria, but unbalanced relaxation by topoisomerase activity due to either inhibition or mutation will lead to alteration in the local level of DNA supercoiling (12, 13). Thus the leu-500 promoter might be activated by negative supercoiling arising from the transcription of the putative nearby gene, which would be less efficiently relaxed in topA cells.

Although this model could explain the activation of the chromosomal leu-500 promoter in topA Salmonella, a further complication came to light when we sought to reproduce the effect on a plasmid. We found that we could only obtain significant activity of the leu-500 promoter when the plasmid also bore the gene encoding resistance to tetracycline, tetA. Using such plasmids we could achieve topA-dependent activation of the promoter in either Salmonella (10) or Escherichia coli (14). This implied a key role for the tetA gene, and a number of studies have indicated that the coupled transcription, translation, and membrane insertion of the tetA gene product are essential for efficient oversupercoiling of plasmids in topA eubacterial cells (13, 15–17) due to the anchorage of the transcribing RNA polymerase to the membrane. We showed that activation of the leu-500 promoter on a plasmid did indeed require transcription and translation of the tetA gene and insertion of the TetA polypeptide into the membrane (10, 18).

We can conceive of two roles for the tetA gene in the activation of the leu-500 promoter on a plasmid. First, transcription of the tetA gene could be the primary generator of supercoiling; tethering RNA polymerase to the membrane would be a particularly effective way in which to hinder its rotation about the DNA template, and thus efficient induction of supercoiling might be expected. The second role could be more passive: to provide a barrier to the diffusion of supercoiling. If negative and positive domains of supercoiling were generated by transcription elsewhere on the plasmid, these could diffuse around the circle and cancel each other by rotation about the duplex axis, providing a highly efficient nonenzymatic relaxation mechanism. However a point of anchorage (such as the insertion of the nascent TetA polypeptide into the membrane) should provide a barrier to the diffusion of supercoiling around the plasmid and might thus increase local levels of DNA supercoiling.

In the plasmid pLEU500Tc, with which we first achieved the topA-dependent activation of the leu-500 promoter (10), the
Activation of the leu-500 Promoter

**TABLE I**

List of plasmids used in this work

| Plasmid Name | Description |
|--------------|-------------|
| pLEU500Tc    | Original plasmid containing leu-500 promoter and clockwise tetA |
| pL500TR      | Fully functional anticlockwise tetA |
| pL500TR.DP_{tet-cov} | pL500TR with deletion of anticlockwise tetA promoter |
| pL500TR.Tet48 | pL500TR with translation terminator in NheI site of anticlockwise tetA |
| pL500TR.Tet96 | pL500TR with translation terminator in Sall site of anticlockwise tetA |
| pL500TR.Tet188 | pL500TR with translation terminator in NruI site of anticlockwise tetA |
| pL500TR.Tet296 | pL500TR with 30% N-terminal deletion of bla |
| pL500TR.Delta | pL500TR with deletion of clockwise tetA promoter |
| pL500TR.Delta | pL500TR with deletion of bla and clockwise tetA promoter |
| pL500TR.Tet48 | pL500TR with deletion of clockwise and anticlockwise tetA promoters |
| pL500TR.Bla12 | pL500TR with translation terminator in Eco57 site of bla |
| pL500TR.Bla80 | pL500TR with translation terminator in ScaI site of bla |

**Materials and Methods**

**Bacterial Strains and Their Growth Conditions**

E. coli strains HB101 (F·, hsdS20 [r B, m B], rca13, ara-14, proA2, lacY1, galK2, rpsL20 [SmaI], xyl-5, mtl-1, supE44, λ·, and DM800 [topA-cysB204 accA13 gyrB225] (25, 26) have been used in the experiments reported here. Bacteria were cultured at 37 °C with aeration in LB medium or grown on 1.2% LB agar plates. Media were supplemented with antibiotics as required: ampicillin and kanamycin were both used at 50 μg/ml and tetracycline was used at 10 μg/ml (except for strains related to E. coli DM800, where this was reduced to 2 μg/ml tetracycline). E. coli strains were transformed with plasmids using the calcium chloride procedure (27).

**Plasmid Constructions**

The plasmids used in this work are summarized in Table I. pL500TR—The plasmid pLEU500Tc (10) was cleaved with NheI and BalI, and pAT153 (28) was digested with EcoRI and BalI. The NheI and EcoRI terminal were rendered flush by incubation with 2.5 units of balHI endonuclease for 25 min at 37 °C. The blunt-ended DNA was religated to generate a plasmid that contained the modified anticlockwise tetA promoter.

**Extraction and Analysis of Cellular RNA**

RNA was isolated using essentially the method described previously (10). RNA was prepared from 200-μl cultures in the mid-exponential growth phase by the addition of an equal volume of 20 mM sodium acetate (pH 5.2), 2% SDS, 0.3 mM sucrose, and transferring to a boiling water bath for 1 min. The sample was then extracted twice with phenol/ chloroform, and the nucleic acids were precipitated with ethanol. After the addition of 0.2 pmol of the appropriate radioactively [5^32P]-labeled DNA probe, the sample was heated to 90 °C in 4.5 μl of 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and rapidly cooled. 25 units of RNase (0.5 μl) were added, and the solution was incubated at 43 °C for 20 min before the addition of 12 μl of 70% tris-HCl (pH 8.0), 70 mM KCl, 15 mM MgCl₂, 15 mM dithiothreitol, 1.3 mM deoxyoxygenoside triphosphate mixture containing 50 units of moloney murine leukemia virus reverse transcriptase (Superscript Plus, Life Technologies, Inc.) and incubated at 42 °C for 2 h. DNA transcripts were electrophoresed in 8% polyacrylamide gels in 90 mM Tris borate (pH 8.3), 10 mM EDTA (TBE) containing 7 M urea, next to sequence markers generated by dideoxy sequence reactions (29) using the same primer. After drying the gels, radioactive fragments were visualized by autoradiography at −70 °C with intensifier screens or with storage phosphor screens and a 400 S PhosphorImager (Molecular Dynamics). Quantitation of radioactivity was performed directly upon the phosphorimage using ImageQuant (Molecular Dynamics).

**Analysis of Linking Number of Extracted Plasmid DNA**

E. coli cells were grown in 30 ml of LB plus appropriate antibiotics to mid-exponential growth phase, and the plasmid DNA was extracted using the Wizard Plus DNA extraction system (Promega). The purified DNA was electrophoresed in 1% agarose gels in TBE containing 2 μg/ml chloroquine. After electrophoresis, the gels were subjected to extensive washing in water for 2 h, 1 μl of ethidium bromide and further washing in water. The stained gels were photographed under UV illumination with red and green filters to remove background fluorescence. The photograph negatives were scanned electronically, and a negative image was presented.

**RESULTS**

Reversal of the tetA Gene of pLEU500Tc—In previous studies we showed that the activation of the leu-500 promoter on the...
plasmid pLEU500Tc in \( \top A \) \( S. \) \( \text{typhimurium} \) was dependent on the function of the adjacent tetracycline resistance gene \( \text{tetA} \) (10). The orientation of the \( \text{tetA} \) gene in pLEU500Tc is opposite to that of the \( \text{leu-500} \) promoter, i.e., the \( \text{leu-500} \) promoter is located immediately upstream of the \( \text{tetA} \) gene. Thus transcription of \( \text{tetA} \) might be the major generator of negative supercoiling in this local region, by the mechanism of Liu and Wang (11). Activation of the \( \text{leu-500} \) promoter in pLEU500Tc required the coupled transcription and translation of \( \text{tetA} \) and the membrane insertion of its product (10, 18). This suggested that membrane insertion of the TetA protein was essential to provide an anchorage point, which might act as a topological barrier against the diffusion of DNA supercoiling. These two related yet distinct roles for the tetA gene might be dissected if its polarity were reversed in the plasmid, and we therefore constructed a new plasmid pL500TR that contains a tetA gene oriented anticlockwise in the conventional depiction of pBR322-based plasmids. The reversed tetA gene is fully functional, and transformed cells have normal levels of resistance to tetracycline. pL500TR still contains the original clockwise \( \text{tetA} \) promoter, but the gene (\( \text{tetA}^- \)) is truncated at the 48th codon. It also contains the anticlockwise antitet promoter. The plasmid map of pL500TR is shown in Fig. 1.

**topA-dependent Activation of the leu-500 Promoter of pL500TR—**In our earlier study, we demonstrated topA-dependent activation of the \( \text{leu-500} \) promoter on plasmid pLEU500Tc containing a clockwise \( \text{tetA} \) gene. To investigate the effect of a reversed polarity tetA gene on the activity of the \( \text{leu-500} \) promoter, RNA was isolated from pL500TR-carrying \( \top A \) \( E. \) \( \text{coli} \) cells in mid-exponential growth, and transcripts initiated at the \( \text{leu-500} \) promoter were sought. This was achieved by means of run-off reverse transcription using a primer that hybridizes to the vector sequence upstream of the \( S. \) \( \text{typhimurium} \) DNA (10). A cDNA corresponding to RNA initiated at the \( \text{leu-500} \) promoter should be 191 nucleotides in length. Since the antitet promoter (the tetR promoter transcribing the same strand as the \( \text{leu-500} \) promoter) is retained on pL500TR, cDNA corresponding to initiation at this promoter would be 281 nucleotides in length and provides a useful reference for quantitation.

The results of the reverse transcription analysis are shown in Fig. 2A. There is a clear band of cDNA corresponding to initiation at the \( \text{leu-500} \) promoter in DM800 (\( \Delta \text{topA} \)) cells, but the intensity of this species is very much lower for the RNA extracted from SD108 (\( \top^d \)). The cDNA band corresponding to initiation at the antitet promoter is of similar intensity in both \( \top^d \) and \( \Delta \text{topA} \) experiments. Thus the \( \text{leu-500} \) promoter was activated by the reversed polarity tetA gene, and this activation was dependent on the \( \Delta \text{topA} \) background.

The activity of the \( \text{leu-500} \) promoter as a function of the polarity of the tetA gene is directly compared in Fig. 2B using cells carrying either pLEU500Tc or pL500TR. RNA was extracted from \( E. \) \( \text{coli} \) DM800 (\( \Delta \text{topA} \)) in exponential growth and subjected to reverse transcription analysis as before. The level of initiation at the \( \text{leu-500} \) promoter is closely similar in both plasmids. Thus the \( \text{topA} \)-dependent activation of the \( \text{leu-500} \) promoter does not depend on the orientation of the tetA gene.
significant reduction by the promoter deletion in the reversed tetA promoter. Thus the topA-dependent activation of the leu-500 promoter in pL500TR requires transcription of the reversed tetA gene.

Activation of the leu-500 Promoter Requires Translation of the Reversed tetA Gene—By analogy with the role of the clockwise tetA gene of pLEU500Tc, it seemed probable that translation would be required in the reversed gene of pL500TR. This was examined by provoking premature termination of translation of the reversed tetA gene by introducing translation terminators at various positions in the coding sequence. This was achieved by introducing complementary oligonucleotides into the NheI, BamHI, SstI, and NruI restriction sites along the tetA gene, thereby generating truncated TetA polypeptides of 48, 96, 188, and 296 amino acids, respectively. These can be compared with the full-length TetA that is 394 amino acids in length. These plasmids are called pL500TR.Tet48, pL500TR.Tet96, pL500TR.Tet188, and pL500TR.Tet296, respectively.

These plasmids were transformed into E. coli DM800 (ΔtopA), RNA was prepared from cells in exponential growth, and the initiation of transcription from the leu-500 promoter was analyzed by reverse transcription as before. Electrophoretic analysis of the cDNA (Fig. 4A) showed that the level of activity of the leu-500 promoter became lower as the length of the TetA polypeptide synthesized. The data were quantified by phosphorimaging and are presented graphically in Fig. 4B. Evidently the function of the leu-500 promoter is dependent on translation of the reversed tetA gene, and the level of the activation of the leu-500 promoter is approximately linearly dependent on the size of the TetA polypeptide synthesized. Thus the topA-dependent activation of the leu-500 promoter depends both on transcription and translation of the reversed tetA gene. This closely parallels the situation where the tetA gene was oriented clockwise in the original construct pLEU500Tc, suggesting that a similar mechanism of activation of the leu-500 promoter is involved in both cases.

Negative Supercoiling of Reversed-tetA Plasmids Isolated from ΔtopA E. coli—When plasmids carrying a functioning tetA gene are isolated from topA E. coli or S. typhimurium in exponential growth and their linking number distribution examined by electrophoresis in agarose gels containing the intercalator chloroquine, it is generally observed that there is a bimodal distribution of topoisomers, one fraction of which is very highly negatively supercoiled. We have previously shown this to be the case for pLEU500Tc and demonstrated a corre-
significant distance from the other more local promoters are important in the generation of

It is possible that the primary role is the latter function and that the negative supercoiling. Since the promoter of the reversed tetA gene is a site of hypersupercoiling, we therefore turned our attention to other gene expression occurring within the vicinity of the leu-500 promoter. This arises primarily from the bla gene and the original tetA gene of which the promoter is retained in pL500TR.

To determine the effect of local gene expression on the activity of the leu-500 promoter, a number of new plasmids were constructed. pL500TR.Δp

These plasmids were transformed into E. coli DM800 (ΔtopA), cellular RNA was isolated from cells in mid-exponential growth, and the initiation of transcription at the leu-500 promoter was analyzed by primer extension as before (Fig. 6A).

The activity of the leu-500 promoter in pL500TR.Δp

Effect of Premature Termination of Translation of the bla Gene on the Activation of the leu-500 Promoter in pL500TR—Previous experiments showed that in the original construct with a clockwise tetA gene (pLEU500TC), initiation of transcription at the leu-500 promoter was influenced by translation of the bla gene under some circumstances (18). We therefore examined the effect of modulating the function of the bla gene on the activity of the leu-500 promoter in the presence of the reversed tetA gene. Two new plasmids were constructed to examine the influence of bla translation. pL500TR.Bla12 and pL500TR.Bla80 contain translation termination codons inserted into the bla coding sequences at the Eco57 and ScaI sites, respectively, generating β-lactamase polypeptides shortened from 263 amino acids to 12 or 80 amino acids, respectively.

The plasmids were transformed into DM800 (ΔtopA), cellular RNA was isolated from cells in mid-exponential growth, and the initiation of transcription at the leu-500 promoter was analyzed by reverse transcription as before (Fig. 6B). The activity of the leu-500 promoter was not significantly reduced in either of these plasmids, indicating that translation of the bla gene is relatively unimportant in the activation of the leu-500 promoter in pL500TR.

FIG. 5. Topoisomer distributions of pL500TR and derivatives extracted from toipa E. coli. Plasmid DNA was isolated from DM800 in exponential growth, and topoisomers were separated by electrophoresis in agarose in the presence of chloroquine. pL500TR (lane 6) exhibits a typical bimodal distribution of topoisomers, with a hypersupercoiled fraction of DNA (indicated at the right). This was compared with the plasmids containing the translation terminators at various positions in the reversed tetA gene and with the plasmid containing the deletion of the promoter of the reversed tetA gene. The DNA was visualized by staining in ethidium bromide and photographed under UV light. A negative image is presented. Lane 1, pL500TR.Tet96; lane 2, pL500TR.Tet96; lane 3, pL500TR.Tet188; lane 4, pL500TR.Tet48; lane 5, pL500TR.Δp

lation between the degree of activation of the leu-500 promoter and the extent of this hypersupercoiled fraction (14). We therefore examined the plasmids carrying the reversed tetA gene to see if these were similarly subject to hypersupercoiling.

Plasmid DNA was isolated from E. coli DM800 (ΔtopA) in exponential growth and analyzed by electrophoresis in 1% agarose in TBE buffer containing 2 μg/ml chloroquine (Fig. 5). The distribution of pL500TR topoisomers was clearly bimodal, with a significant fraction of hypersupercoiled DNA. Reversing the polarity of the tetA gene has not changed its effect on the overall topology of the plasmid. Interference with the function of the tetA gene reduces the extent of this fraction of highly supercoiled plasmid. The proportion was severely reduced for pL500TR.Δp

These plasmids were transformed into E. coli DM800 (ΔtopA), cellular RNA was isolated from cells in mid-exponential growth, and the initiation of transcription at the leu-500 promoter was analyzed by primer extension as before (Fig. 6A). The activity of the leu-500 promoter in pL500TR.Δp

By contrast, as we have seen above, deletion of the promoter of the reversed tetA gene (lane 7) results in a marked reduction in activity of the leu-500 promoter, and combination of the deletions of both tetA promoters results in a very similar low level of leu-500 promoter activity (lane 8). We conclude that the dominant effector of the topA-dependent activation of the leu-500 promoter is the reversed tetA gene.

Effect of Premature Termination of Translation of the bla Gene on the Activation of the leu-500 Promoter in pL500TR—Previous experiments showed that in the original construct with a clockwise tetA gene (pLEU500TC), initiation of transcription at the leu-500 promoter was influenced by translation of the bla gene under some circumstances (18). We therefore examined the effect of modulating the function of the bla gene on the activation of the leu-500 promoter in the presence of the reversed tetA gene. Two new plasmids were constructed to examine the influence of bla translation. pL500TR.Bla12 and pL500TR.Bla80 contain translation termination codons inserted into the bla coding sequences at the Eco57 and ScaI sites, respectively, generating β-lactamase polypeptides shortened from 263 amino acids to 12 or 80 amino acids, respectively.

The plasmids were transformed into DM800 (ΔtopA), cellular RNA was isolated from cells in mid-exponential growth, and the initiation of transcription at the leu-500 promoter was analyzed by reverse transcription as before (Fig. 6B). The activity of the leu-500 promoter was not significantly reduced in either of these plasmids, indicating that translation of the bla gene is relatively unimportant in the activation of the leu-500 promoter in pL500TR.
**Activation of the leu-500 Promoter**

Our results clearly demonstrate that the leu-500 promoter can be activated on a plasmid in topA E. coli by the presence of a tetA gene in either orientation. Activation requires the full function of the tetA gene, but the leu-500 promoter can be located in a position that can be regarded either as primarily upstream or one that is downstream of this gene. Moreover, the role of the tetA gene is paramount; although other promoters present in pL500TR are of relatively minor consequence, inactivation of tetA function reduces the activity of the leu-500 promoter to background levels. In summary, the tetA gene is essential for the topA-dependent activation of the leu-500 promoter, but its orientation is unimportant.

It might be regarded as surprising that this effect is independent of tetA orientation; that the activation of the leu-500 promoter is equally efficient when it is placed in what is formally the domain of positive supercoiling (downstream of tetA) (11), as when it is located in the upstream domain of negative supercoiling. We therefore change our perspective from a local view of variation in superhelicity to a more global view. The local view supposes that the leu-500 promoter must be located directly within the domain of negative supercoiling to be activated. In the global view, unbalanced relaxation of transcriptional-induced supercoiling from the tetA gene results in a net reduction in the linking difference of the plasmid. If the tetA gene is the primary generator of supercoiling (because of its membrane anchoring effect), then it will create local domains of negative and positive supercoiling. If only the latter can be relaxed in a topA cell, the overall effect will be to lower the linking number of the plasmid. If the leu-500 promoter is responding to this global change in topology, then it will do so independent of relative orientation or separation.

We arrive at the same conclusion following a second line of argument. As we discussed in the introduction, an alternative role of membrane anchorage by coupled transcription, translation, and insertion of TetA could be to provide a topological barrier so that the domains of positive and negative supercoiling generated by transcription (in theory from any promoter) cannot diffuse around the circular plasmid and undergo self-cancellation by a simple rotation of the helix. If this were true, it would require the existence of a second barrier on the opposite side of the circular plasmid, and it has been suggested that the replication origin might function in this way (19). The combined effect of two such barriers would effectively isolate the lower half of the plasmid in topological terms. However, in pL500TR, the promoter of the reversed tetA gene would be located in this domain, isolated topologically from the leu-500 promoter. Yet we have shown that the single most important factor on the plasmid for the topA-dependent activation of the leu-500 promoter is the tetA promoter. We therefore conclude that it cannot be located in a separate domain and that the barrier model does not hold. We are left with the primary role of membrane anchorage as the provision of rotational hindrance to RNA polymerase transcribing the tetA gene. Since the tetA and leu-500 promoters are separated by more than 1.6

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**FIG. 6.** Effect of local gene expression on the activity of the leu-500 promoter. A, effect of local gene activity of the leu-500 promoter. Deletions within the bla gene and the promoters of the truncated tetA gene and reversed tetA gene of pL500TR were made in various combinations. These were transformed into E. coli DM800 (ΔtopA) and RNA isolated from cells in exponential growth. Initiation of transcription at the antitet and leu-500 promoters was analyzed by reverse transcription and electrophoretic separation of cDNA products. The positions of cDNA species corresponding to mRNA initiated at the antitet and leu-500 promoters are indicated on the right. Note that the cDNA product corresponding to initiation at the antitet promoter in the ΔPtet constructs is shortened due to the deletion introduced into the template. Lanes 1 and 2 contain sequence markers generated by C and G dideoxy sequencing reactions, respectively. Lane 3, RNA isolated from cells carrying pL500TR; lane 4, RNA isolated from cells carrying pL500TRΔbla; lane 5, RNA isolated from cells carrying pL500TRΔPtet; lane 6, RNA isolated from cells carrying pL500TRΔPtetΔPtopA; lane 7, RNA isolated from cells carrying pL500TRΔPtetΔPtopAΔPσ70; lane 8, RNA isolated from cells carrying pL500TRΔPtetΔPtopAΔPσ70ΔPσ70. B, effect of β-lacta-

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**DISCUSSION**

Our results clearly demonstrate that the leu-500 promoter can be activated on a plasmid in topA E. coli by the presence of a tetA gene in either orientation. Activation requires the full function of the tetA gene, but the leu-500 promoter can be located in a position that can be regarded either as primarily upstream or one that is downstream of this gene. Moreover, the role of the tetA gene is paramount; although other promoters present in pL500TR are of relatively minor consequence, inactivation of tetA function reduces the activity of the leu-500 promoter to background levels. In summary, the tetA gene is essential for the topA-dependent activation of the leu-500 promoter, but its orientation is unimportant.

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We arrive at the same conclusion following a second line of argument. As we discussed in the introduction, an alternative role of membrane anchorage by coupled transcription, translation, and insertion of TetA could be to provide a topological barrier so that the domains of positive and negative supercoiling generated by transcription (in theory from any promoter) cannot diffuse around the circular plasmid and undergo self-cancellation by a simple rotation of the helix. If this were true, it would require the existence of a second barrier on the opposite side of the circular plasmid, and it has been suggested that the replication origin might function in this way (19). The combined effect of two such barriers would effectively isolate the lower half of the plasmid in topological terms. However, in pL500TR, the promoter of the reversed tetA gene would be located in this domain, isolated topologically from the leu-500 promoter. Yet we have shown that the single most important factor on the plasmid for the topA-dependent activation of the leu-500 promoter is the tetA promoter. We therefore conclude that it cannot be located in a separate domain and that the barrier model does not hold. We are left with the primary role of membrane anchorage as the provision of rotational hindrance to RNA polymerase transcribing the tetA gene. Since the tetA and leu-500 promoters are separated by more than 1.6
The global view of the activation is consistent with measurement of the linking difference of isolated plasmids (e.g., Fig. 5), which is a measure of the local topology by definition. This shows that the fraction of hypersupercoiled plasmid DNA is generated whenever the tetA gene is present in cis, whatever its orientation. Indeed, we obtain a linear correlation between the level of activation of the leu-500 promoter in topA E. coli with the fraction of hypersupercoiled plasmid DNA isolated from the cells (Fig. 7). In situ probing of the formation of cruciform structures by alternating adenine-thymine ((AT)ₙ) sequences can be used as a means of testing local negative superhelix density in cellular DNA (20), and we have shown that reporter (AT)ₙ sequences introduced in the region corresponding to that upstream of tetA in pLEU500Tc detect unconstrained oversupercoiling in topA strains (21). However, contrary to initial expectations, we also detected elevated negative supercoiling at (AT)ₙ sequences placed downstream of the tetA gene, i.e., in the region that might be expected to experience transcriptional induction of positive supercoiling. Once again this result is more consistent with a global view of the induction of negative plasmid supercoiling in topA cells.

The topA-dependent activation of the leu-500 promoter in pL500TR does differ in some respects from that in the original pLEU500Tc containing the clockwise tetA gene. One is the effect of bla expression; we observed that bla deletion lowered the level of leu-500 promoter activation in pLEU500Tc (18), whereas there is little influence of bla in the presence of the anticlockwise tetA gene of pL500TR. However, we found that the effect of bla deletion on the leu-500 promoter in pLEU500Tc could be removed when a tac promoter was introduced into this plasmid, suggesting that subtle effects may occur in this region. Another difference is the effect of spacing. When we introduced random DNA fragments between the leu-500 and tetA promoters of pLEU500Tc, this reduced the level of initiation of transcription at the former, whereas in pL500TR, the crucial Pₜₐₓₑᵦ, is almost diametrically opposite to the leu-500 promoter. At present we are unable to account for this difference.

There have been reports of activation of the leu-500 promoter in topA cells using plasmids that do not include the tetA gene (22, 23). We find these observations perplexing, because in our experiments the role of the tetA gene is paramount. It is conceivable that other factors play a role in these constructs, but it is possible that the overall level of activation of transcriptional initiation was lower in those investigations. It is beyond question that in the plasmids based upon pLEU500Tc, the role of the tetA gene is essential for the observed level of activation and cannot be replaced by any other gene that we have explored. Moreover, correlation with the physical level of hypersupercoiling in our plasmids has been independently confirmed by the experiments of Mojica and Higgins (24), who measured the level of unconstrained plasmid supercoiling using an intercalation assay.

In summary, the leu-500 promoter is activated highly efficiently in topA cells when it is borne on a plasmid carrying the tetA gene in cis, irrespective of orientation. The most probable explanation is that it is activated by negative supercoiling arising from the transcription of the tetA gene and that this process is most effective when RNA polymerase is effectively tethered due to coordinate transcription, translation, and membrane insertion. The coupling between the promoters can be fully explained by topological effects operating within the plasmid globally.

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