[Frontiers in Bioscience 8, 430-439, January 1, 2003]

TRANSCRIPTION-DRIVEN DNA SUPERCOILING AND GENE EXPRESSION CONTROL

Chien-Chung Chen, and Hai-Young Wu

Department of Pharmacology, School of Medicine, Wayne State University, Detroit, Michigan 48201, USA

TABLE OF CONTENTS

1. ABSTRACT

DNA supercoiling plays important roles in gene expression regulation, although, the underlying mechanisms whereby DNA supercoiling modulates gene expression remain elusive. The fact that the transcription process itself generates DNA supercoiling has further complicated the issue. Transcription-driven DNA supercoiling is local and transient. Such a DNA supercoiling effect is likely to play important roles in controlling complex gene expression regulation. Using the suppression of the *leu-500* mutation in *Salmonella typhimurium topA* mutants as a model system, we put forward our view of the effects of transcription-driven DNA supercoiling on gene expression control.

2. INTRODUCTION

Studies have shown that DNA supercoiling of the duplex DNA template is perturbed in many helical tracking processes such as the elongation of RNA polymerase complex (1), the activity of simian virus 40 (SV40) large T tumor antigen (2), and the tracking *E. coli UvrAB* complex (3). The possibility of forming such fluctuating DNA supercoiling was first proposed by Liu and Wang in 1987 when DNA topological constraints were put into a model of the elongation process of RNA polymerase complex during transcription (4). Since then, the DNA supercoiling concept has gained support from various *in vivo* and *in vitro* studies using both prokaryotic and eukaryotic model systems (1, 5-7). According to the twin-domain model of transcription (4), positive supercoiling is generated in front of, and negative supercoiling behind, a moving RNA polymerase complex due to the helical rotation of the DNA template (illustrated in Figure 1). The anticipated DNA helix rotation during the tracking process of RNA polymerase has recently been visualized using real-time optical microscopy (8). While many identified (2, 3) and not-yet-identified (9) helical tracking proteins are capable of generating a similar DNA supercoiling fluctuation in the DNA template, the most significant source of DNA supercoiling fluctuation on the chromosome in a living cell is likely to be the helical tracking of the RNA polymerase complex as transcription activity is so abundant. At a given location on the chromosome, the DNA supercoiling dynamic is expected to be temporally associated with the gene activity in the region. During the transcription process, the local DNA supercoiling will be perturbed and the re-distribution of DNA supercoiling will be dependent on the chromosomal architecture (supercoiling barriers) in the local region. Hence, the superhelical state of chromosome DNA is highly dynamic rather than static. Such fluctuations in DNA supercoiling are expected to be very significant in an active chromosome where

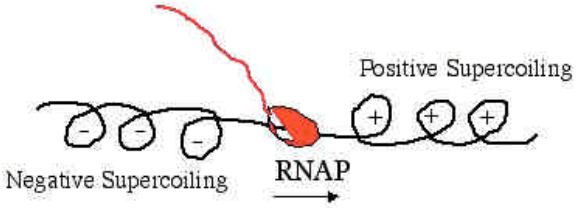

Figure 1. Transcription-driven DNA supercoiling. During the transcription elongation, positive supercoiling is generated in front of, and negative supercoiling behind, the moving RNA polymerase complex (RNAP).

transcription machineries are functioning as "factories" at the fixed transcription foci (10). According to the twin-domain model of transcription (4), an anchored RNA polymerase complex drives DNA supercoiling at a maximum.

The DNA supercoiling dynamic driven by the transcription activities on the chromosome provides a mechanistic basis to understand the functional relationships between transcription and other DNA supercoiling-sensitive cellular processes on the chromosome (reviewed in 11). A DNA supercoiling-sensitive cellular process at a particular chromosome location is presumably affected by the DNA supercoiling fluctuation driven by gene activity in the local region. Transcription-driven DNA supercoiling has been demonstrated as a potential factor in the regulation of recombination (12, 13) and replication (14). Interestingly, transcription-driven DNA supercoiling is capable of affecting the transcription processes themselves since transcription initiation (the open complex formation) is a DNA supercoiling-sensitive process (15). Numerous studies have demonstrated the effect of transcription-driven DNA supercoiling on transcription control using various gene expression model systems. Examples are: the expression of *Escherichia coli ilvYC* operon (16-18); the transcription initiation of N4 virion RNA polymerase promoters (19-23); Xenopus rRNA transcription *in vivo* (24, 25); the regulation of chloroplast gene expression in Chlamydomonas (26); and the regulation of human *c-myc* expression (27, 28 & reviewed in 29). DNA supercoiling effects are therefore apparently common in various gene systems, and the supercoiling effects are likely to be responsible for aspects of the spatial and temporal precision of gene expression control that are not fully explicable with the classical Monod and Jacob's genetic control concept (30).

To understand the underlying mechanisms responsible for the *leu-500* activation phenomenon in *Salmonella typhimurium topA* mutants, we have unraveled an interesting promoter relay mechanism whereby the expression of genes in the *ilvIH-leuO-leuABCD* gene cluster is coordinated in a sequential manner. Transcription-driven DNA supercoiling is likely to provide signals at various stages during the gene expression coordination. Several transcription elements in the region were found to be responsive to adjacent transcription activities. Such responsiveness is presumably due to the DNA supercoiling driven by the neighboring transcription activities and could be one of the underlying mechanisms whereby expression of neighboring genes is coordinated. Inclusion of the effects of transcription-driven DNA supercoiling into studies of this gene expression system has thus revealed a new class of transcription regulatory element that is responsive to transcription-driven DNA supercoiling. Further elucidating the molecular details of the new elements may therefore shed light on our understanding of the effects of DNA supercoiling on gene expression control.

3. SUPPRESSION OF THE *LEU-500* MUTATION IN *S. TYPHIMURIUM TOPA* MUTANTS: A DNA SUPERCOILING CORRELATION NOW AND THEN

3.1. The DNA supercoiling correlation for the suppression of the *leu-500* mutation in *S. typhimurium topA* mutants: A straightforward explanation

The *leu-500* mutation results in leucine auxotrophy in *S. typhimurium* (31). The mutation abolishes the promoter activity of the *S. typhimurium* leucine operon due to an A to G transition in the -10 sequence of the promoter. Subsequently, second-site mutations, suppressor X, that restore the leucine prototrophy were found (32). This second mutation site was later identified to be the DNA topoisomerase I gene (*topA*) (33, 34). Since then, the suppression of the *leu-500* mutation in *S. typhimurium topA* mutants has been one of the paradigms for demonstrating the importance of negative DNA supercoiling on transcription initiation. The GC base pair (vs. the wild-type A·T base pair) in the *leu-500* promoter is expected to increase the energy barrier for the open complex formation at the mutant promoter. Negative supercoiling on the DNA template is important to overcome such an energy barrier. The absence of TopA is known to cause hyper-negative DNA superhelicity in *topA* mutants (34). The higher free energy state of the hyper-negative DNA supercoiling in *topA* mutants had been the straightforward explanation for why the transcription activity of the impaired (*leu-500*) promoter was restored in *topA* mutants (33, 34). Based on this explanation, transcription initiation from the *leu-500* promoter had been expected to correlate with the degree of the negative superhelicity on the DNA template.

3.2. The deficiency of the straightforward explanation

The straightforward DNA supercoiling explanation was tested in 1984. In the study, suppression of the *leu-500* mutation was monitored in a series of *S. typhimurium topA* mutants with combinations of $topA^+$ or $topA^-$ and various compensatory *gyrA* or *gyrB* mutations (35). At the same time, the overall DNA superhelicity was measured in the DNA extracted from the *topA* mutant series. Surprisingly, the suppression of the *leu-500* mutation correlated only with the absence of TopA and did not correlate with the increase of the overall DNA negative superhelicity. This result clearly indicated that the activation of the supercoiling-sensitive *leu-500* promoter in *topA* mutants was not simply due to the overall increase in DNA negative superhelicity in the $topA^-$ genetic background as previously suggested (34). However, the absence of TopA (null mutation of *topA*) is somehow

absolutely required for the *leu-500* activation. This requirement suggested that local rather than global DNA supercoiling was important for the *leu-500* activation (35).

More strikingly, *topA*$^-$ dependent suppression of the *leu-500* mutation occurs only when the *leu-500* promoter is located at its original chromosomal location. The *leu-500* promoter (position -80 to +87 of the *leu* operon) was subcloned onto an extrachromosomal DNA and the plasmid was then transformed into the *S. typhimurium topA* mutant that carries the chromosomal *leu-500* mutation. While the chromosomal *leu-500* promoter was activated as expected, the plasmid-borne *leu-500* promoter failed to be activated in the *topA*$^-$ strains (36). This finding suggested that an unknown regulatory element(s), which was essential for the DNA supercoiling-dependent transcriptional activation, was left behind on the chromosome (not present between positions -80 and +87 of the minimal promoter DNA sequence) during the subcloning. However, no upstream regulatory elements were found in a search up to position -857 of the 5' region upstream from the leucine operon (36). The form and location of the crucial regulatory element(s) responsible for the activation of the supercoiling-dependent *leu-500* promoter in the chromosome context remained a mystery.

3.3. The new explanation

The above studies have elegantly demonstrated that the underlying mechanism responsible for the phenomenon of suppression of the *leu-500* mutation in *topA* mutants is more complex than the previously suggested straightforward DNA supercoiling explanation (34). It had been puzzling that the activation of *leu-500* promoter correlates only with the absence of TopA (35). Since the activity of *leu-500* promoter is DNA supercoiling-sensitive, whatever factor responsible for the *leu-500* activation is most likely to generate negative DNA supercoiling locally. The demonstration of local supercoiling fluctuation driven by transcription activity (1) has led to a hypothesis that this may be the missing element that provides the crucial negative DNA supercoiling locally for the *leu-500* activation. In a review article (37), Lilley and Higgins suggested the possible involvement of an adjacent transcription activity in the activation of the DNA supercoiling-sensitive *leu-500* promoter.

This hypothesis was tested in several studies (38-41). The plasmid-borne *leu-500* promoter (position -80 to +87 of the *leuABCD* operon) was indeed activated when a transcription activity leading away from the *leu-500* promoter minimal sequence was provided. Most importantly, this activation was dependent on the *topA*$^-$ genetic background. The *topA*$^-$ genetic background dependency is consistent with the correlation between the activation of the *leu-500* promoter and the absence of TopA (35). While several parameters such as a divergent pair of transcription units and *tetA* gene product mediated membrane anchorage were shown to be required for the activation of the plasmid-borne *leu-500* promoter in studies from Lilley's group (38, 39), our result indicated that a simple transcription activity that transcribes away from the *leu-500* promoter is sufficient to result in activation of the promoter (40). Sprito and Bossi later confirmed that membrane anchorage was not required for the *leu-500* activation in their study (41). In our testing, the activation induced by adjacent transcription away from the *leu-500* promoter is limited to a short-range. Depending on the strength of the promoter, the adjacent promoter has to be positioned within from 250 bp to 450 bp from the *leu-500* promoter (40). We named this activation as "short-range promoter-promoter interaction" (40, 42).

According to the twin-domain model of transcription (4), many parameters may affect the accumulation of transcription-driven DNA supercoiling. The two conditions in Dr. Lilley's studies: a divergent pair of transcription units, and the *tetA* gene product mediated membrane anchorage, are parameters that facilitate DNA supercoiling accumulation. Since the activation of *leu-500* promoter is a DNA supercoiling sensitive indicator, the promoter is certainly activated by the accumulated DNA supercoiling more readily. As far as the underlying mechanism for the suppression of the *leu-500* mutation in *topA* mutants is concerned, the relevant fact here is that transcription-driven DNA supercoiling may be the missing element responsible for the activation of the chromosomal *leu-500* promoter. In fact, this is the only logical conclusion that can be drawn from studies where the minimal *leu-500* promoter DNA sequence position (-80 to +87 of the *leuABCD* operon) is surrounded with the rather foreign plasmid DNA context.

4. LONG-RANGE PROMOTER-PROMOTER INTERACTION

Based on the above argument for a possible missing element, we searched for transcription activity that we predicted to be located upstream of the *leuABCD* operon. Graduated extension of the upstream chromosome DNA region in the plasmid-borne *leu-500* promoter revealed that activation occurred when the promoter of the *ilvIH* operon was also included (42). The *ilvIH* operon is located 1.9 kb upstream and its transcription away from the *leuABCD* operon (see illustration in Figure 2). Mutation or deletion of the *ilvIH* promoter abolished the *leu-500* activation. Replacement of the *ilvIH* promoter with an IPTG-inducible *lac* promoter resulted in IPTG-dependent *leu-500* activation (42). Hence the evidence is compelling in that it is the promoter activity rather than the specific promoter that is important for the *leu-500* activation. The involvement of the upstream promoter activity in the *leu-500* activation suggested strongly that transcription-driven DNA supercoiling is important for the long-range (1.9 kb) promoter-promoter interaction.

This was a rather surprising finding since the previous results where *leu-500* promoter was surrounded with the plasmid DNA context (40) had indicated that the effect of transcription-driven DNA supercoiling on *leu-500* activation was limited to a short-range. In contrast, when part of the intervening 1.9 kb DNA sequence was deleted, the *ilvIH* promoter-mediated transcription activity was no longer able to activate *leu-500* promoter despite the fact that the distance between the two interacting promoters was

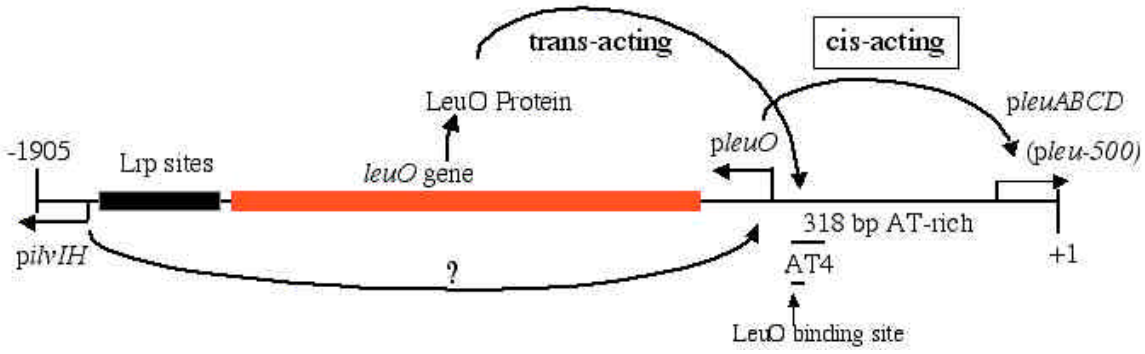

Figure 2. The *ilvIH-leuO-leuABCD* gene locus where promoter relay is at work. The presently known elements located upstream of *S. typhimurium leuABCD* operon (+1 to –1905 position of the operon) are illustrated. The mechanism by which *ilvIH* promoter-mediated transcription activity activates the intermediated *leuO* gene in the first place remains unknown (the question mark). The gene silencer and LeuO binding site in the AT4 DNA segment may be important to explain why the *leuO* gene is normally silent and conditionally activated upon the activation of *ilvIH*.

shortened by the deletion (42). This discrepancy suggested strongly that while transcription-driven DNA supercoiling may be the basis for both short-range (40) and long-range (42) promoter-promoter interactions, the underlying mechanisms responsible for the two interactions must be very different.

Indeed, further testing indicated that unlike the *topA*$^-$genetic background dependency of the short-range promoter-promoter interaction (40), the long-range promoter-promoter interaction was not absolutely dependent on the *topA*$^-$ genetic background (42). Both experiments were carried out on plasmids. The major difference between the two testing conditions was that the *leu-500* promoter minimal DNA sequence was embedded in the foreign plasmid DNA context in the first case (40), with the two interacting promoters divided by plasmid DNA. In the latter case, the entire 1.9 kb upstream chromosome DNA fragment was associated with the *leu-500* promoter (42) and so chromosomal DNA is present between the two interacting promoters. In the presence of the native chromosomal DNA context, the *leu-500* activation was no longer dependent on the *topA*$^-$ genetic background. It was, however, highly dependent on the integrity of the chromosome DNA context. Apparently, the simple activation model (40) based on the transcription-driven DNA supercoiling is insufficient to explain the long-range interaction. A more complex mechanism must be involved in the long-range promoter-promoter interaction for relaying the DNA supercoiling effect over the long (1.9 kb) distance.

4.1. A promoter relay mechanism is responsible for the long-range promoter-promoter interaction

Since the 1.9 kb intervening DNA sequence is important for the long-range promoter-promoter interaction, we searched for the possible sequence elements in the 1.9 kb region. Based on the available information from literature, three elements were identified: the Lrp (leucine-responsive regulatory protein) binding sites (43), an open reading frame (ORF) of the putative *leuO* gene (44), and a stretch of AT-rich DNA sequence upstream of *leuABCD* operon (45) (illustrated in Figure 2). The Lrp binding sites located immediately upstream of *ilvIH* operon is presumably the regulatory region for controlling the expression of *ilvIH* operon. The intermediate *leuO* gene was hypothetical since no transcription activity responsible for the expression of the gene was found in an earlier attempt (45). The AT-rich DNA sequence is flanked by the divergently arrayed *leuO* gene and *leuABCD* operon and presumably contains the transcription regulatory elements important for the promoter-promoter interaction since deletion of this AT-rich DNA sequence abolished the long-range promoter-promoter interaction (42).

We searched for the possible transcription activity responsible for the middle gene since this putative *leuO* gene is a candidate of transcription activity that could relay the DNA supercoiling effect over the 1.9 kb distance. In a series of primer extension studies, we found one transcription initiation site upstream of the ORF. This newly identified transcription activity was strikingly dependent on the *ilvIH* transcription activity (46). This finding suggested that *leuO* transcription activity could relay the DNA supercoiling effect initially triggered by the *ilvIH* promoter-mediated transcription. A computer program (MacVector by Accelrys Inc.) predicted a promoter DNA sequence located upstream of the detected transcription initiation site. The predicted –10 and –35 DNA sequences separated by an 18-bp spacer fit the consensus promoter sequence for sigma^{70} *E. coli* RNA polymerase (47, 48). We mutated the promoter by introducing a 2-bp mutation at the –10 DNA sequence of the predicted promoter, and we found that the 2-bp mutation at the promoter abolished the long-range promoter-promoter interaction while the *ilvIH* promoter activity remained intact (46). This result confirms that this promoter activity indeed was responsible for relaying the DNA supercoiling effect over the 1.9 kb distance.

More detailed analysis indicated that both the gene product and the promoter activity of the intermediate

leuO gene are important for the subsequent activation of *leu-500* promoter. It was then clear that the transcription activity of the *ilvIH* promoter somehow activated the intermediate *leuO* gene. The *leuO* gene product, LeuO, plays a trans-acting role and *leuO* promoter activity plays a cis-acting role for the final activation of the *leu-500* promoter. This sequential gene activation process was named as promoter relay mechanism (46). While many details in the promoter relay wait to be elucidated, the promoter relay mechanism explains the long-range interaction between *ilvIH* and *leu-500* promoters and provides a model whereby neighboring genes are communicating with each other via transcription-driven DNA supercoiling.

4.2. A topA$^-$ genetic background is not absolutely required for the promoter relay mechanism

The discovery of the promoter relay mechanism derived from the phenomenon of suppression of the *leu-500* mutation, which was concluded to be absolutely dependent on the absence of TopA (35). Surprisingly, therefore, the promoter relay mechanism that is apparently responsible for the long-range interaction between *ilvIH* and *leu-500* promoters is not absolutely dependent on the *topA*$^-$ genetic background (42). This discrepancy prompted us to clarify the relationship between TopA and the promoter relay mechanism. By monitoring the mRNA of the normally silent *leuO* gene, we found that the promoter relay mechanism indeed occurred in the presence of TopA (49). It appears that the transcription activity mediated by the *ilvIH* promoter plays a decisive role in the promoter relay and that TopA plays a negative regulatory role in the promoter relay since the presence of TopA only resulted in 2-fold reduction of *leu-500* activation that is triggered by *ilvIH* promoter activity (49).

Suppression of the *leu-500* mutation (leucine prototrophy) had been described in the *S. typhimurium topA*$^-$ mutants such as CH582 (35, 36). Due to the leucine auxotrophic phenotype (the *leu-500* mutation and the wild-type *topA* genetic background) in CH601, the parental strain of CH582, the leucine auxotrophic CH601 strain was not expected to grow in a leucine-free medium if, as previously suggested, a *topA*$^-$ genetic background is absolutely required for the activation of the *leu-500* promoter in CH582. If, on the other hand, a *topA*$^-$ genetic background is not absolutely required for the *leu-500* activation via the promoter relay mechanism, we predicted that as long as the *ilvIH* gene was turned on and thus triggered the promoter relay mechanism, *S. typhimurium* CH601 strain should be able to grow in a leucine-free medium because of the activation of the *leu-500* promoter. Indeed, after a prolonged lag phase, CH601 grew in a leucine-free medium (49). Northern blotting analysis indicated that the transcription activities of both normally silent *ilvIH* and *leuO* became detectable prior to the time when CH601 cells resumed their growth after the prolonged (16 hour) lag phase. Western blotting analysis indicated the TopA level remained constant throughout the growth of CH601, including during the 16-hour lag phase (49). Hence, it was clear that under the stress (the prolonged lag phase), *ilvIH* transcription was somehow turned on and triggered the promoter relay mechanism that resulted in the *leu-500* activation in the *topA* wild-type CH601 cells. This finding strikingly decoupled the *leu-500* activation and the *topA*$^-$ genetic background requirement at a chromosomal DNA level. It seems that *ilvIH* transcription activity-triggered promoter relay is a stress response in the bacterium regardless of its *topA* genetic background. This possibility also gains support from a finding that *ilvIH* promoter activity is under the positive control of Lrp (43, 50), which is a global transcription regulator whose cellular level is up-regulated by cellular guanosine 3', 5'-bispyrophosphate (ppGpp) in response to the reduction of growth rate caused by nutrient limitation (51).

4.3. A new explanation for the *leu-500* activation phenomenon in *topA* mutants

The results, thus far, have clearly shown that *topA*$^-$ genetic background is not absolutely required for the promoter relay mechanism that is presumably responsible for the *leu-500* activation in *topA*$^-$ mutants such as CH582. Thus it is important to understand why *leu-500* activation had been tightly associated with the absence of TopA (35, 36). The link of the promoter relay with the reduction of growth rate in response to nutrient limitation (51) has prompted us to consider the fact that *topA*$^-$ mutants such as CH582 have much slower growth rates than *topA* wild-type strains. The slow growth rate reflects the growth stress in *topA*$^-$ mutants due to the absence of TopA. Could this be one of the stresses that cause the activation of *ilvIH* and subsequently trigger the promoter relay mechanism in CH582? Northern blotting analysis demonstrated that the *ilvIH* operon was indeed constantly active during the entire growth of CH582 (49). So it is now clear that the correlation of the *leu-500* activation phenomenon with the absence of TopA (35, 36) is due to the promoter relay mechanism mediated sequential activation of genes in the *ilvIH-leuO-leuABCD* gene cluster in response to the growth stress associated with the *topA* mutants. While the molecular details remain to be further elucidated, the promoter relay mechanism has provided answers to explain several previous mysterious observations made during the almost 4-decade long research effort toward understanding the fascinating *leu-500* activation phenomenon in *S. typhimurium topA* mutants.

5. THE PROMOTER RELAY MECHANISM AS A STRESS RESPONSE IN BACTERIA

It appears that the promoter relay is a gene regulatory process in response to growth stress rather than a specific event limited to a phenomenon in *topA* mutants. Is it physiologically important in bacteria?

5.1. As part of the normal cell physiology, the promoter relay mechanism is a ppGpp-dependent stress response

The ppGpp-Lrp pathway (51) is proposed to be responsible for the activation of *ilvIH* operon, which then subsequently triggers the promoter relay mechanism under growth stress. In normal bacterial physiology, ppGpp serves as a stress signal in the bacterial stringent response pathway. The signal, ppGpp, was also shown to be

important for the bacterial cell survival in the stationary phase (reviewed in 52). To elucidate the potential ppGpp link of the promoter relay, we have identified two conditions when normally silent *leuO* expression is induced via the promoter relay mechanism. The transcription regulatory machinery appears to turn on when bacterial cells are entering stationary phase. The transient induction of *leuO* expression at the late-log/stationary phase is not dependent on the stationary phase-specific *rpoS* (sigma-s factor). Instead, this transient *leuO* expression is very sensitive to the biosynthesis of ppGpp, the upstream signal of *rpoS* expression (53).

The second conditional *leuO* induction was found when bacterial cells are under extreme starvation for branched-chain amino acids during exponential growth in a chemically defined medium. In response to such severe branched-chain amino acid depletion, a *leuO*$^-$ phenotype (cells failed to resume their growth after the 2 hr growth arrest during log phase) was found during the growth of *leuO* knocked-out *E. coli relA1* strain (54). The expression of *leuO* was transiently induced via the promoter relay mechanism during the 2 hr growth arrest. This conditional *leuO* expression appears to be part of the bacterial stringent response triggered by ppGpp. The promoter relay mechanism is controlled decisively by the transcription activity of *ilvIH* (46). The expression of *ilvIH* is presumably activated via the ppGpp-Lrp pathway (51). The slow onset of ppGpp in the "relaxed" *relA1* strains is expected to make the *ilvIH* remain "repressed" in the *E. coli relA1* strain. There is conclusive evidence that the "relaxed" *relA1* strain was at a physiological impasse (the 2-hr growth arrest) from which escape was possible by the derepression of the repressed genes (the *ilv* operons and hence *leuO* via the promoter relay mechanism) (54).

The two conditional *leuO* expression conditions strongly suggested that the promoter relay is part of the stringent response triggered by nutrient limitation during bacterial growth. We also provided evidence that LeuO is important for bacterial cells to make a proper decision on their continuous cell division under the nutrient limitation-mediated growth arrest (54). It seems that *leuO* is one of those genes that are not important under normal laboratory growth condition (usually rich medium is used), but is crucial for bacteria to survive in nature where environmental stresses including nutrient-limitation are widespread.

5.2. The physiological implication of the promoter relay mechanism

The physiological importance of the promoter relay in the *ilvIH-leuO-leuABCD* gene region is compelling. The promoter relay mechanism was unraveled when monitoring the on-off activity of the mutant *leu-500* promoter of the *leuABCD* operon (42, 46). We had demonstrated that the wild-type *leuABCD* promoter is only affected by the promoter relay mechanism approximately three-fold (46). Based on our finding of the specific physiological function of the promoter relay mechanism in response to the starvation for branched-chain amino acids (54), it is, however, reasonable that such a three-fold increase may be critical for cell survival under the severe branched-chain amino acid starvation. The *ilvIH* operon encodes AHASIII (acetohydroxy acid synthase III) required for the synthesis of precursors for the downstream biosynthesis of branched-chain amino acids including the biosynthesis of leucine catalyzed by the gene products of *leuABCD* operon. Hence, under a severe branched-chain amino acid starving condition, the communication between the *ilvIH* operon and the *leuABCD* operon via the promoter relay mechanism may be one of the important physiological functions of the promoter relay mechanism. Such gene communication may be crucial for the efficient production of the life-saving leucine in cells under starvation for branched-chain amino acids (54).

The second physiological importance of the promoter relay mechanism is related to the fact that *leuO* gene is located between the two communicating operons in response to starvation of branched-chain amino acid. As part of the promoter relay mechanism, the expression of *leuO* is fully repressed when cells are not under stress (e.g. nutrient limitation). In a rich broth, the expression of *leuO* is almost not detectable at both the mRNA and protein levels (53, 54). Such tight control explains the failure of an earlier attempt of finding the transcription activity responsible for the, then, putative *leuO* gene (45). The tight associations of *ilvIH* expression with the ppGpp-Lrp pathway (51) and our findings regarding the stringent response to the starvation for branched-chain amino acid (54) have strongly suggested a nutrient limitation stress sensor role of the *ilvIH* expression. Via the promoter relay mechanism, the *leuO* expression is induced by the transcription activity of *ilvIH* operon in response to environmental stresses.

The position of *leuO* between the two genes that are responsive to growth stress may not be a coincidence. During the 2 hr growth arrest caused by the starvation of branched-chain amino acid, LeuO was shown to play indispensable role(s) to determine the subsequent cell division (cell growth resumption). Cell division is a complex cell event. Hence, besides the obvious function for LeuO at the *ilvIH-leuO-leuABCD* gene cluster site to increase the expression of the *leuABCD* operon for three-fold as the final activation step in the promoter relay mechanism, LeuO may act *in trans* to impact cell physiology by affecting the expression of other genes as well. We have demonstrated, using two-dimensional protein gel electrophoresis, that the expression of at least 27 genes in *E. coli* are affected by LeuO either positively or negatively upon its induction during the starvation for branched-chain amino acid (Wu, unpublished data). Whether all or some of these 27 genes are directly affected by LeuO remains to be investigated. Such a prominent transcription regulatory effect of LeuO may be responsible for its striking effect on cell division (54).

6. THE TRANSCRIPTION REGULATORY FUNCTION OF LEUO

The importance of a global transcription regulatory effect of LeuO has been supported by a number of studies using various *E. coli* gene systems. LeuO is

involved in controlling the expression of the regulatory DsrA-RNA (55, 56). LeuO affects the expression of the acid-inducible lysine decarboxylase gene (57). LeuO also exerts both positive and negative effects on the silencing of the expression of the *bgl* operon (58). Potential LeuO regulons are located at various positions throughout the bacterial chromosome including the *leuABCD* operon. LeuO thus appears to be a transcription regulator that trans-actingly affects the expression of many genes. To understand the transcription regulatory function of LeuO, we have investigated the LeuO function in the *ilvIH-leuO-leuABCD* gene cluster. In the investigation, we have revealed two regulatory elements, a 47-bp gene silencer sequence and LeuO, that are important for the gene expression coordination in the gene cluster. Both elements are located upstream of the *leuO* gene and are presumably important for controlling the repression of *leuO* gene and its transient activation as part of the promoter relay mechanism. Since transcription-driven DNA supercoiling has been the basis for explaining the sequential gene activation in the promoter relay mechanism, we were not surprised to find that the transcription regulatory function of the gene silencer is highly dependent on the activity of the adjacent transcription units (59). Such a functional responsiveness to the adjacent transcription activities may be the key feature for these transcription regulatory elements to coordinate expression between genes.

6.1. Bacterial gene silencer AT4 mediated transcriptional repression

To elucidate the molecular details whereby the expression of genes in the *ilvIH-leuO-leuABCD* gene cluster are coordinated via the promoter relay mechanism, we investigated why LeuO coupled with the transcription activity of *leuO* promoter (p*leuO*) is required for the final step of gene activation (the *leu-500* activation) in the promoter relay mechanism (46). The 318-bp intervening DNA sequence between the divergently arrayed *leuO* and *leuABCD* (see illustration in Figure 2) is AT rich (69 % A+T). The function of this AT-rich DNA sequence was unknown. Deletion of this AT-rich DNA sequence severely impaired the long-range interaction between *ilvIH* and *leu-500* promoters (42). This finding suggested that the AT-rich DNA is functionally important for the promoter relay mechanism. Initial tests indicated that this AT-rich DNA is transcriptionally repressive. The transcription repression activity has been narrowed down to a 72-bp AT-rich (78% A+T) DNA named AT4, which is located at the *leuO* end of the 318-bp AT-rich intervening DNA (illustrated in Figure 2). AT4 DNA-mediated transcriptional repression is promoter nonspecific, orientation independent, and additive. Based on the striking general transcription repression activity, we suggested that the 72-bp AT4 DNA functions as a bacterial gene silencer (59). The gene silencing effect can reach up to a distance of 300 base pairs from the target promoter. Interestingly, the gene silencing activity is very sensitive to the condition of the flanking transcription units. The gene silencer functions optimally when it is flanked by a pair of divergent transcription units that both transcribe away from the gene silencer. This positional effect is consistent with the natural location of the gene silencer, which is situated between the divergently arrayed *leuO* and *leuABCD* (see illustration in Figure 2). We explained this striking positional effect of gene silencer in terms of DNA supercoiling driven by the flanking transcription activities (59).

6.2. LeuO functions as a derepression element, which negates the gene silencer-mediated transcriptional repression.

LeuO appears to target directly on the AT4 DNA and to negate AT4-mediated transcriptional repression (59). In detailed analyses designed to reveal the functional relationship between the silencer and LeuO, we successfully divided the 72-bp AT4 DNA into two functional elements, the gene silencer AT8 and the LeuO binding site AT7. The 47-bp gene silencer AT8 retained the full gene silencing activity of AT4. The 25-bp AT7 LeuO binding site contains no gene silencing activity. LeuO binding within the proximity negates the gene silencer AT8-mediated transcriptional repression (Chen & Wu unpublished data). To understand the underlying mechanism responsible for the striking transcriptional derepression, the 40 kDa LeuO protein has been analyzed biochemically. LeuO associates with some yet to be identified proteins to form a complex that apparently causes a DNA structural transition at the sequence adjacent to the LeuO binding site. Such LeuO-mediated DNA structural transition appears to be very sensitive to the adjacent transcription activities as well (Wu, unpublished data).

While the molecular details remained to be elucidated in the promoter relay mechanism, a key feature is apparently responsible for the expression coordination of genes in the *ilvIH-leuO-leuABCD* gene cluster. The critical event is the obvious involvement of transcription activity at the various stages during the sequential gene activation. The responsiveness of transcription elements, gene silencer and LeuO, to adjacent transcription activities may provide the answers to the question of how communication occurs to provide coordination of transcription through sending transcription-driven DNA supercoiling.

7. CONCLUDING REMARKS

Transcription-driven DNA supercoiling has been the basis of our explanation for the series of interesting findings in this model system. Indeed, many previously difficult to understand observations are clarified by including the transcription-driven DNA supercoiling in the current model. Further, transcription factors with novel, interesting features were found based on their involvement in transcription-driven DNA supercoiling. However, it has proven difficult to demonstrate directly the presence of transcription-driven DNA supercoiling in this regulation. So far, the involvement of transcription-driven DNA supercoiling in such regulation has been based on the presence of an active promoter. Due to the transient nature of transcription-driven DNA supercoiling, a better methodology has to be developed to directly monitor its presence.

Nonetheless, the effect of transcription-driven DNA supercoiling on gene expression control is

compelling. Results from Dr. Levens' group and Dr. Rothman-Denes' group have elegantly demonstrated the involvement of various DNA structural elements in the gene expression control of *c-myc* (reviewed in 29) and in the transcription regulation at the N4 virion promoters (reviewed in 20). It appears that unusual DNA structures are capable of regulating transcription including gene expression on chromatin (reviewed in 60). The important lesson we learned from the *leu-500* activation model system was that it is the local, transient DNA supercoiling (e.g. transcription-driven DNA supercoiling) rather than the overall accumulated DNA supercoiling (e.g. the measurable average DNA supercoiling on a reporter plasmid) that is important for regulating gene expression. The transcription elements that undergo DNA structural transitions (reviewed in 20, 29, & 60) may share the same underlying mechanism whereby the transcription elements (gene silencer AT8 and LeuO) are responsive to transcription-driven DNA supercoiling as a local and transient cellular event.

8. PERSPECTIVE

Both gene silencer AT8 and LeuO binding site AT7 are located in the 318-bp AT-rich intervening DNA sequence between the divergently arrayed *leuO* and *leuABCD* in *S. typhimurium* (see illustration in Figure 2). The AT-rich DNA sequence was one of the important factors that drew our attention to the *ilvIH-leuO-leuABCD* gene locus in the first place. The *ilvIH-leuO-leuABCD* gene cluster in the two closely related enteric bacteria, *S. typhimurium* and *E. coli*, are similarly positioned. While the DNA sequences of the coding regions of the involved genes showed high homology between the two microorganisms, the DNA sequence homology is low in the AT-rich DNA flanking by the *leuO* and *leuABCD* genes. The only homology of the intervening DNA segments between the two closely related bacteria is the AT-richness (45). It is clear that this AT-rich intervening DNA sequence is the transcription regulatory region for the promoter relay mechanism since the two important transcription regulatory elements are located in this region. Despite the lack of DNA sequence homology, the regulatory elements on the *S. typhimurium* DNA can be recognized by *E. coli* protein factors for the promoter relay mechanism. This is suggested by the observation that the plasmid born *S. typhimurium* promoter relay DNA sequence (NCBI accession numbers AF106956) functions normally in various *E. coli* hosts and vice versa (Wu, unpublished data). This result strongly suggested that it is DNA structure rather than the specific DNA sequence that is important for the regulation in the promoter relay mechanism. Indeed, our recent studies toward identifying the similar transcription elements in the *E. coli* promoter relay region (NCBI accession numbers AF106955) have indicated that the narrowed down gene silencer and LeuO binding site are also present in *E. coli* but have no DNA sequence homology with their counter parts found in *S. typhimurium* (59). Strikingly, the *E. coli* and *S. typhimurium* elements are functionally interchangeable (Wu, unpublished data). Hence, it is evidenced that DNA structural elements in the region are responsible for the regulation in the promoter relay mechanism. This is consistent with the fact that the functions of these transcription regulatory elements are responsive to local transcription-driven DNA supercoiling. With the transcription-driven DNA supercoiling emphasized, a transcription regulatory mechanism involving DNA structural transition is emerging from the elucidation of the sequential activation of genes in the *ilvIH-leuO-leuABCD* gene locus. Many interesting mechanistic details will be unraveled in future elucidations of the conserved functions of the AT-rich DNA elements that are functionally similar but lacking DNA sequence homology in *E. coli* and *S. typhimurium*.

9. ACKNOWLEDGMENTS

We are in debt to Dr. Ray Mattingly for his critical reading of the manuscript. This work is supported by National Institutes of Health, National Institute of General Medical Sciences, grant# GM-53617. We would also like to express our highest appreciation to Dr. Leroy F. Liu for his encouragement and moral support along the entire course of this research. The research is build upon a foundation consists of results of many elegant studies. We appreciate the accumulated efforts made by many people in this research field. In particular, we appreciate Drs. David Lilley, Joseph Calvo, Wesley Hatfield, Regine Henggna-Aronis, and Chiharu Ueguchi for the numerous helpful discussions and/or providing materials.

10. REFERENCES

1. Wu, H. Y., S. H. Shyy, J. C. Wang, & L. F. Liu: Transcription generates positively and negatively supercoiled domains in the template. *Cell* 53, 433-440 (1988)

2. Yang, L., C. B. Jessee, K. Lau, H. Zhang, & L. F. Liu: Template supercoiling during ATP-dependent DNA helix tracking: studies with simian virus 40 large tumor antigen. *Proc Natl Acad Sci USA* 86, 6121-6125 (1989)

3. Koo, H. S., L. Claassen, L. Grossman, & L. F. Liu: ATP-dependent partitioning of the DNA template into supercoiled domains by *Escherichia coli UvrAB. Proc Natl Acad Sci U S A* 88, 1212-1216 (1991)

4. Liu, L. F. & J. C. Wang: Supercoiling of the DNA template during transcription. *Proc Natl Acad Sci USA* 84, 7024-7027 (1987)

5. Tsao, Y. P., H. Y. Wu, & L. F. Liu: Transcription-driven supercoiling of DNA: direct biochemical evidence from *in vitro* studies. *Cell* 56, 111-118 (1989)

6. Brill, S. J. & R. Sternglanz: Transcription-dependent DNA supercoiling in yeast DNA topoisomerase mutants. *Cell* 54, 403-411 (1988)

7. Giaever, G. N. & J. C. Wang: Supercoiling of intracellular DNA can occur in eukaryotic cells. *Cell* 55, 849-856 (1988)

8. Harada, Y., O. Ohara, A. Takatsuki, H. Itoh, N. Shimamoto, & K. Kinosita, Jr.: Direct observation of DNA rotation during transcription by *Escherichia coli* RNA polymerase. *Nature* 409, 113-115 (2001)

9. Zhang, H., C. B. Jessee, & L. F. Liu: A protein factor from Xenopus oocytes with simian virus 40 large tumor antigen-like DNA supercoiling activity. *Proc Natl Acad Sci USA* 87, 9078-9082 (1990)

10. Cook, P. R.: The organization of replication and transcription. *Science* 284, 1790-1795 (1999)
11. Droge, P.: Protein tracking-induced supercoiling of DNA: a tool to regulate DNA transactions *in vivo*? *Bioessays* 16, 91-99 (1994)
12. Droge, P.: Transcription-driven site-specific DNA recombination *in vitro*. *Proc Natl Acad Sci U S A* 90, 2759-2763 (1993)
13. Wang, Z. & P. Droge: Long-range effects in a supercoiled DNA domain generated by transcription *in vitro*. *J Mol Biol* 271, 499-510 (1997)
14. Asai, T., C. P. Chen, T. Nagata, M. Takanami, & M. Imai: Transcription *in vivo* within the replication origin of the *Escherichia coli* chromosome: a mechanism for activating initiation of replication. *Mol Gen Genet* 231, 169-178 (1992)
15. Borowiec, J. A. & J. D. Gralla: All three elements of the *lac* ps promoter mediate its transcriptional response to DNA supercoiling. *J Mol Biol* 195, 89-97 (1987)
16. Rhee, K. Y., M. Opel, E. Ito, S. Hung, S. M. Arfin, & G. W. Hatfield: Transcriptional coupling between the divergent promoters of a prototypic LysR-type regulatory system, the *ilvYC* operon of *Escherichia coli*. *Proc Natl Acad Sci USA* 96, 14294-14299 (1999)
17. Opel, M. L. & G. W. Hatfield: DNA supercoiling-dependent transcriptional coupling between the divergently transcribed promoters of the *ilvYC* operon of *Escherichia coli* is proportional to promoter strengths and transcript lengths. *Mol Microbiol* 39, 191-198 (2001)
18. Opel, M. L., S. M. Arfin, & G. W. Hatfield: The effects of DNA supercoiling on the expression of operons of the *ilv* regulon of *Escherichia coli* suggest a physiological rationale for divergently transcribed operons. *Mol Microbiol* 39, 1109-1115 (2001)
19. Rothman-Denes, L. B., X. Dai, E. Davydova, R. Carter, & K. Kazmierczak: Transcriptional regulation by DNA structural transitions and single-stranded DNA-binding proteins. *Cold Spring Harb Symp Quant Biol* 63, 63-73 (1998)
20. Dai, X. & L. B. Rothman-Denes: DNA structure and transcription. *Curr Opin Microbiol* 2, 126-130 (1999)
21. Dai, X., M. Kloster, & L. B. Rothman-Denes: Sequence-dependent extrusion of a small DNA hairpin at the N4 virion RNA polymerase promoters. *J Mol Biol* 283, 43-58 (1998)
22. Dai, X. & L. B. Rothman-Denes: Sequence and DNA structural determinants of N4 virion RNA polymerase-promoter recognition. *Genes Dev* 12, 2782-2790 (1998)
23. Dai, X., M. B. Greizerstein, K. Nadas-Chinni, & L. B. Rothman-Denes: Supercoil-induced extrusion of a regulatory DNA hairpin. *Proc Natl Acad Sci USA* 94, 2174-2179 (1997)
24. Dunaway, M. & E. A. Ostrander: Local domains of supercoiling activate a eukaryotic promoter *in vivo*. *Nature* 361, 746-748 (1993)
25. Krebs, J. E. & M. Dunaway: DNA length is a critical parameter for eukaryotic transcription *in vivo*. *Mol Cell Biol* 16, 5821-5829 (1996)
26. Salvador, M. L., U. Klein, & L. Bogorad: Endogenous fluctuations of DNA topology in the chloroplast of *Chlamydomonas reinhardtii*. *Mol Cell Biol* 18, 7235-7242 (1998)
27. Tomonaga, T. & D. Levens: Activating transcription from single stranded DNA. *Proc Natl Acad Sci U S A* 93, 5830-5835 (1996)
28. Michelotti, G. A., E. F. Michelotti, A. Pullner, R. C. Duncan, D. Eick, & D. Levens: Multiple signle-stranded *cis* elements are associated with activated chromatin of the human *c-myc* gene *in vivo*. *Mol cell Biol* 16, 2656-2669 (1996)
29. Levens, D., R. C. Duncan, T. Tomonaga, G. A. Michelotti, I. Collins, T. Davis-Smyth, T. Zheng, & E. F. Michelotti: DNA conformation, topology, and the regulation of *c-myc* expression. *Curr Top Microbiol Immunol* 224, 33-46 (1997)
30. Jacob, F.: Genetics of the bacterial cell. *Science* 152, 1470-1478 (1966)
31. Mukai, F. H., & P. Margolin: Analysis of unlinked suppressors of an *Oo* mutation in *Salmonella*. *Proc. Natl. Acad. Sci. USA* 50, 140-148 (1963)
32. Dubnau, E. & P. Margolin: Suppression of promoter mutations by the pleiotropic *supx* mutations. *Mol Gen Genet* 117, 91-112 (1972)
33. Trucksis, M., E. I. Golub, D. J. Zabel, & R. E. Depew: *Escherichia coli* and *Salmonella typhimurium supX* genes specify deoxyribonucleic acid topoisomerase I. *J Bacteriol* 147, 679-681 (1981)
34. Margolin, P., L. Zumstein, R. Sternglanz, & J. C. Wang: The *Escherichia coli supX* locus is *topA*, the structural gene for DNA topoisomerase I. *Proc Natl Acad Sci USA* 82, 5437-5441 (1985)
35. Richardson, S. M., C. F. Higgins, & D. M. Lilley: The genetic control of DNA supercoiling in *Salmonella typhimurium*. *Embo J* 3, 1745-1752 (1984)
36. Richardson, S. M., C. F. Higgins, & D. M. Lilley: DNA supercoiling and the *leu-500* promoter mutation of *Salmonella typhimurium*. *Embo J* 7, 1863-1869 (1988)
37. Lilley, D. M. & C. F. Higgins: Local DNA topology and gene expression: the case of the *leu-500* promoter. *Mol Microbiol* 5, 779-783 (1991)
38. Chen, D., R. Bowater, C. J. Dorman, & D. M. Lilley: Activity of a plasmid-borne *leu-500* promoter depends on the transcription and translation of an adjacent gene. *Proc Natl Acad Sci USA* 89, 8784-8788 (1992)
39. Chen, D., R. P. Bowater, & D. M. Lilley: Activation of the *leu-500* promoter: a topological domain generated by divergent transcription in a plasmid. *Biochemistry* 32, 13162-13170 (1993)
40. Tan, J., L. Shu, & H. Y. Wu: Activation of the *leu-500* promoter by adjacent transcription. *J Bacteriol* 176, 1077-1086 (1994)
41. Spirito, F., N. Figueroa-Bossi, & L. Bossi: The relative contributions of transcription and translation to plasmid DNA supercoiling in *Salmonella typhimurium*. *Mol Microbiol* 11, 111-122 (1994)
42. Wu, H. Y., J. Tan, & M. Fang: Long-range interaction between two promoters: activation of the *leu-500* promoter by a distant upstream promoter. *Cell* 82, 445-451 (1995)
43. Wang, Q., M. Sacco, E. Ricca, C. T. Lago, M. De Felice, & J. M. Calvo: Organization of Lrp-binding sites upstream of *ilvIH* in *Salmonella typhimurium*. *Mol Microbiol* 7, 883-891 (1993)
44. Henikoff, S., G. W. Haughn, J. M. Calvo, & J. C. Wallace: A large family of bacterial activator proteins. *Proc Natl Acad Sci USA* 85, 6602-6606 (1988)
45. Haughn, G. W., S. R. Wessler, R. M. Gemmill, & J. M. Calvo: High A + T content conserved in DNA sequences upstream of *leuABCD* in *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol* 166, 1113-1117 (1986)

46. Fang, M. & H. Y. Wu: A promoter relay mechanism for sequential gene activation. *J Bacteriol* 180, 626-633 (1998)
47. Siebenlist, U., R. B. Simpson, & W. Gilbert: *E. coli* RNA polymerase interacts homologously with two different promoters. *Cell* 20, 269-281 (1980)
48. Kirkegaard, K., H. Buc, A. Spassky, & J. C. Wang: Mapping of single-stranded regions in duplex DNA at the sequence level: single-strand-specific cytosine methylation in RNA polymerase-promoter complexes. *Proc Natl Acad Sci USA* 80, 2544-2548 (1983)
49. Fang, M. & H. Y. Wu: Suppression of *leu-500* mutation in *topA*$^{+}$ *Salmonella typhimurium* strains. The promoter relay at work. *J Biol Chem* 273, 29929-29934 (1998)
50. Willins, D. A. & J. M. Calvo: *In vitro* transcription from the *Escherichia coli ilvIH* promoter. *J Bacteriol* 174, 7648-7655 (1992)
51. Landgraf, J. R., J. Wu, & J. M. Calvo: Effects of nutrition and growth rate on Lrp levels in *Escherichia coli. J Bacteriol* 178, 6930-6936 (1996)
52. Rao, N. N. & A. Kornberg: Inorganic polyphosphate regulates responses of *Escherichia coli* to nutritional stringencies, environmental stresses and survival in the stationary phase. *Prog Mol Subcell Biol* 23, 183-195 (1999)
53. Fang, M., A. Majumder, K. J. Tsai, & H. Y. Wu: ppGpp-dependent *leuO* expression in bacteria under stress. *Biochem Biophys Res Commun* 276, 64-70 (2000)
54. Majumder, A., M. Fang, K. J. Tsai, C. Ueguchi, T. Mizuno, & H. Y. Wu: LeuO expression in response to starvation for branched-chain amino acids. *J Biol Chem* 276, 19046-19051 (2001)
55. Klauck, E., J. Bohringer, & R. Hengge-Aronis: The LysR-like regulator LeuO in *Escherichia coli* is involved in the translational regulation of *rpoS* by affecting the expression of the small regulatory DsrA-RNA. *Mol Microbiol* 25, 559-569 (1997)
56. Repoila, F. & S. Gottesman: Signal transduction cascade for regulation of RpoS: temperature regulation of DsrA. *J Bacteriol* 183, 4012-4023 (2001)
57. Shi, X. & G. N. Bennett: Effects of multicopy LeuO on the expression of the acid-inducible lysine decarboxylase gene in *Escherichia coli*. *J Bacteriol* 177, 810-814 (1995)
58. Ueguchi, C., T. Ohta, C. Seto, T. Suzuki, & T. Mizuno: The *leuO* gene product has a latent ability to relieve *bgl* silencing in *Escherichia coli*. *J Bacteriol* 180, 190-193 (1998)
59. Chen, C. C., M. Fang, A. Majumder, & H. Y. Wu: A 72-base pair AT-rich DNA sequence element functions as a bacterial gene silencer. *J Biol Chem* 276, 9478-9485 (2001)
60. van Holde, K. & J. Zlatanova: Unusual DNA structures, chromatin and transcription. *Bioessays* 16, 59-68 (1994)

Key words: *Leu-500* activation, *topA*, Transcription Control, Transcription-Driven DNA Supercoiling, Bacterial Stress Response, Promoter Relay Mechanism, Review

Send correspondence to: Hai-Young Wu, Department of Pharmacology, Wayne State University, School of Medicine, 540 E. Canfield Ave., Detroit, Michigan 48201, USA. Tel: +1-313-577-1584, Fax:+1-313-577-6739, E-mail: haiwu@med.wayne.edu