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# Activation of transcription initiation from a stable RNA promoter by a Fis protein-mediated DNA structural transmission mechanism

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## Summary

**The *leuV* operon of *Escherichia coli* encodes three of the four genes for the $tRNA_1^{Leu}$ isoacceptors. Transcription from this and other stable RNA promoters is known to be affected by a *cis*-acting UP element and by Fis protein interactions with the carboxyl-terminal domain of the α-subunits of RNA polymerase. In this report, we suggest that transcription from the *leuV* promoter also is activated by a Fis-mediated, DNA supercoiling-dependent mechanism similar to the IHF-mediated mechanism described previously for the *ilv*$P_G$ promoter (S. D. Sheridan *et al*., 1998, *J Biol Chem* 273: 21298–21308). We present evidence that Fis binding results in the translocation of superhelical energy from the promoter-distal portion of a supercoiling-induced DNA duplex destabilized (SIDD) region to the promoter-proximal portion of the *leuV* promoter that is unwound within the open complex. A mutant Fis protein, which is defective in contacting the carboxyl-terminal domain of the α-subunits of RNA polymerase, remains competent for stimulating open complex formation, suggesting that this DNA supercoiling-dependent component of Fis-mediated activation occurs in the absence of specific protein interactions between Fis and RNA polymerase. Fis-mediated translocation of superhelical energy from upstream binding sites to the promoter region may be a general feature of Fis-mediated activation of transcription at stable RNA promoters, which often contain A+T-rich upstream sequences.**

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## Introduction

We have described previously a protein-mediated, DNA structural transmission mechanism that regulates basal level transcription from the *ilv*$P_G$ promoter into the genes of the *ilvGMEDA* operon required for branched-chain amino acid biosynthesis in *Escherichia coli* (Sheridan *et al*., 1998). In this case, the upstream regulatory region of the *ilv*$P_G$ promoter is located in an A+T-rich sequence that defines a supercoiling-induced duplex destabilized (SIDD) region, a site at which moderate levels of negative superhelicity destabilize the DNA duplex. The promoter-distal portion of this SIDD region contains a high-affinity integration host factor (IHF) target binding site. In the absence of IHF binding, negative superhelicity drives duplex destabilization at this position. However, IHF binding forces this region back to the B-form, which transfers the destabilization (negative twist) downstream to the *ilv*$P_G$ promoter elements. This IHF binding-induced transfer of duplex destabilization facilitates strand separation in the –10 region and thereby increases the rate of transcriptional initiation. Several experimental approaches have demonstrated that this mechanism of gene activation by DNA structural transmission occurs in the absence of interactions between IHF and RNA polymerase (Parekh *et al*., 1996; Parekh and Hatfield, 1996; Sheridan *et al*., 1998; 1999).

The *leuV* operon of *Escherichia coli* encodes three of the four genes for the $tRNA_1^{Leu}$ isoacceptors. The *leuV* and other stable RNA operons are transcribed from strong promoters with near-consensus RNA polymerase (RNAP) binding motifs. They are characterized by a G+C-rich discriminator region located between the –10 hexanucleotide binding region and the transcriptional start site (Gourse *et al*., 1996; Keener and Nomura, 1996). Stable RNA promoters are also characterized by the presence of both *cis*- and *trans*-acting activation elements. For example, transcription from the *leuV* promoter is enhanced by a third RNAP recognition element located between basepairs –39 and –47. This A+T-rich UP sequence makes contacts

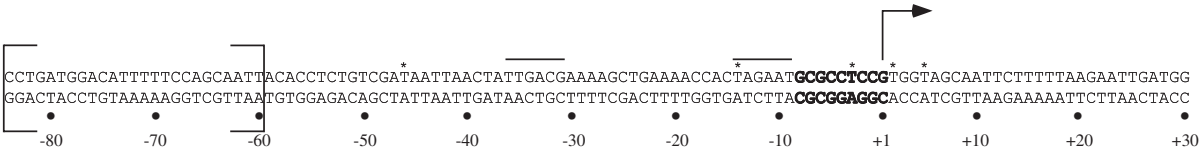


**Fig. 1.** Nucleotide sequence of the *leuV* promoter region from –84 to +30. The transcriptional start site for *leuV* is identified by an arrow. The –10 and –35 hexanucleotide regions are identified by horizontal lines. The Fis binding site is denoted by brackets. The discriminator is identified by bold type. Bases modified by $KMnO_4$ are identified by asterisks.

with the α-subunits of RNAP, stabilizes closed complex formation and activates *leuV* expression more than 10-fold (Ross *et al*., 1993; Estrem *et al*., 1998; Pokholok *et al*., 1999). Further upstream in the *leuV* upstream activating sequence (UAS) region, as in the UAS regions of all stable RNA promoters, there is a Fis protein binding site. Fis binding to this site, centred at basepair position –71, enhances *leuV* expression an additional threefold (Ross *et al*., 1999).

In addition to these regulatory mechanisms, we suggest that the *leuV* promoter is also activated by a Fis-mediated translocation of superhelical energy mechanism similar to the IHF-mediated mechanism described previously for the *ilvP*$_G$ promoter of *E. coli* (Sheridan *et al*., 1998). Like this system, we demonstrate that the *leuV* promoter, as well as its UAS and Fis binding site, are all located in a SIDD region, and investigate the behaviour of this region on a negatively supercoiled DNA template. We show that Fis binding in the promoter-distal portion of this SIDD region is accompanied by increases in both the rate of open complex formation and the rate of transcription from the downstream *leuV* promoter. We suggest that this activation is caused, at least in part, by a Fis binding-mediated translocation of superhelical energy (negative twist) from the promoter-proximal portion of this SIDD domain to the downstream SIDD region containing the *leuV* promoter elements. Finally, we demonstrate that Fis-mediated enhancement of open complex formation is DNA supercoiling dependent and occurs in the absence of specific interactions with the α-subunits of RNAP. Specifically, we show that a mutant Fis protein, which does not make specific contacts with the α-subunits of RNAP, is capable of facilitating open complex in a DNA supercoiling-dependent manner.

## Results

### *A SIDD site is predicted in the* leuV *promoter-regulatory region*

The discriminator region of the *leuV* promoter is located at bp positions –8 to +1 relative to the transcriptional start site (Fig. 1). As it is exceptionally G+C rich, eight of nine bases being either G or C, the discriminator is believed to be a region of strong duplex stability that inhibits open complex formation (Travers and Muskhelishvili, 1998). In order to predict the stability of the discriminator region of the *leuV* promoter under superhelical stress within its experimental context, a DNA SIDD profile was calculated for the 1881 bp portion of the experimental plasmid between the start sites for *bla* and RNA-II which includes the *leuV* promoter. (Benham, 1992; 1993) This region was chosen because it constitutes a topological domain under the experimental conditions.

The results of these calculations are presented in Fig. 2 as destabilization (SIDD) profiles, plots of the incremental free energy $G(x)$ versus position $x$. The figure shows the destabilization of the 400 bp region containing the *leuV* promoter. This SIDD profile predicts that the DNA duplex in the *leuV* promoter-regulatory region from bp positions –94 to +43 is moderately destabilized at a physiological superhelical density of $\sigma = -0.055$ (Fig. 2). As anticipated, there is a sharp peak of strong duplex stability correlating with the discriminator region. An intriguing feature of the SIDD profile is that the Fis target binding site between bp positions –84 to –60 is located in the upstream portion of the SIDD domain.

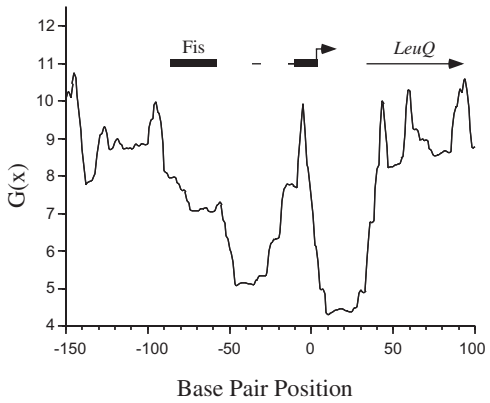


**Fig. 2.** SIDD profile of the *leuV* promoter region from –150 to +100. Predicted free energies $G(x)$ for duplex destabilization at a superhelical density of $\sigma = -0.05$ at base position $x$ are expressed in kcal mol$^{-1}$ bp$^{-1}$. The positions of relevant promoter features are indicated above the SIDD plot. The transcriptional start site is indicated by an arrow. The –10 and –35 hexanucleotide regions are identified by thin horizontal lines. The discriminator is denoted by a thick horizontal line. The Fis binding site and first tRNA$_1^{Leu}$ gene are labelled.

### *Effects of negative DNA supercoiling on transcription from the* leuV *promoter*

The SIDD profile (Fig. 2) suggests a region of strong duplex stability in the discriminator around the *leuV* transcription initiation site. This suggests that high levels of negative DNA supercoiling should be required for open complex formation and, hence, that transcription from the *leuV* promoter should be exceptionally sensitive to increases in negative DNA supercoiling. To test this prediction, *in vitro* transcription reactions were performed on a set of plasmid DNA topoisomer preparations with mean negative superhelical densities ranging from $\sigma = 0.00$ to $\sigma = -0.098$. The results of these experiments showed that, in the absence of Fis, transcription from the *leuV* promoter does indeed increase substantially (90-fold) from its lowest level on a relaxed DNA template to its highest level at a superhelical density of $\sigma = -0.069$, and decreases thereafter (Fig. 3).

### *Fis-mediated translocation of superhelical energy to the* leuV *promoter*

The SIDD profile (Fig. 2) reveals a single Fis binding site overlapping the promoter-distal portion of the SIDD domain. This suggests that Fis binding to this site might stabilize in the B-form the DNA in the upstream portion of the SIDD region. As was documented for the *ilv*$P_G$ promoter, this could translocate local superhelical energy (negative twist) away from the binding site. Translocation of this superhelical energy could further destabilize the DNA duplex around the transcription initiation site, promote open complex formation and thereby activate transcription from the *leuV* promoter.

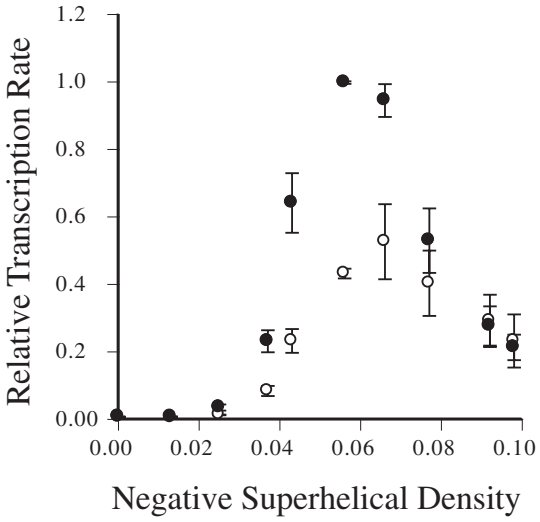


**Fig. 3.** Effects of negative DNA supercoiling on basal level and Fis-activated transcription from the *leuV* promoter. Relative *leuV* transcription rates are plotted as a function of average negative superhelical density in the absence (open circles) and presence (filled circles) of Fis. Values are the mean ± SD of four separate experiments.

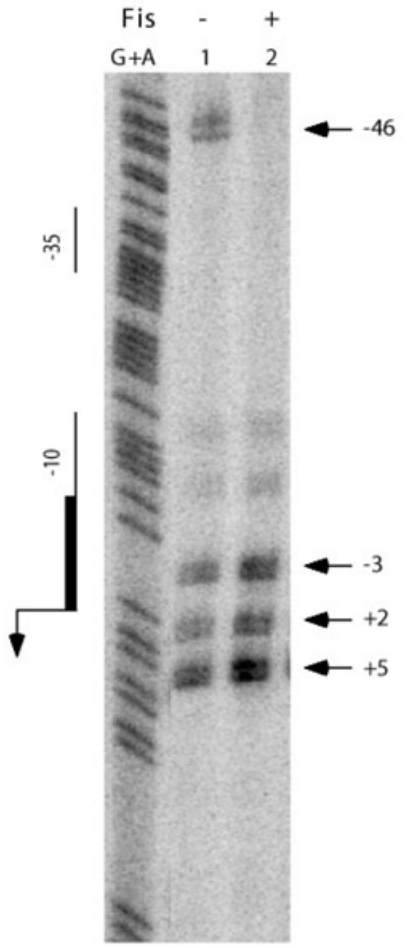


**Fig. 4.** Effect of Fis and RNA polymerase binding on $KMnO_4$ reactivity at the *leuV* promoter. $KMnO_4$ probing was performed in the presence of RNA polymerase and the presence or absence of Fis as described in *Results* and *Experimental procedures*. The coding strand is labelled at the 3′ end. A (G+A) ladder from –54 at the top of the gel to +20 at the bottom of the gel is indicated. Relevant promoter features are indicated as described in the legend to Fig. 2. Arrows indicate positions of $KMnO_4$ reactivity.

To test this prediction, $KMnO_4$ probing of the *leuV* SIDD domain was performed on a plasmid DNA topoisomer preparation with a mean superhelical density of $\sigma = -0.06$. This DNA template was preincubated with RNA polymerase in the presence or absence of Fis for 20 min, followed by a 20 s incubation with the first two initiating nucleotides (G and U), then chemically probed with $KMnO_4$ as described in *Experimental procedures*. In the absence of Fis, $KMnO_4$ reactivity was observed at a thymidine residue located at bp position –46 (Fig. 4, lane 1). This modification was diminished in the presence of a saturating concentration of Fis, indicating a Fis-mediated stabilization of the DNA duplex in the upstream region of

the SIDD domain (Fig. 4, lane 2). At the same time, Fis binding enhanced (1.5- to twofold) the $KMnO_4$ reactivity of three thymidines at bp positions –3, +2 and +5 (Fig. 4, lanes 1 and 2). Thus, Fis binding to its upstream target site on a supercoiled DNA template promotes duplex destabilization near the –10 region of the downstream *leuV* promoter site.

In an experiment performed in the absence of RNA polymerase and initiating nucleotides, we were unable to detect Fis-enhanced $KMnO_4$-sensitive sites in the *leuV* promoter region. We suspect that this is because RNA polymerase binding is required to localize the Fis-induced negative twist to the promoter region (Travers and Muskhelishvili, 1998) and, at the *leuV* promoter as for other stable RNA promoters, stable open complexes cannot be formed in the absence of initiating nucleotides (Gourse, 1988; Gaal *et al*., 1997; Pemberton *et al*., 2000).

### *Fis activation is DNA supercoiling dependent*

To investigate the dependence of Fis-mediated activation on DNA supercoiling, *in vitro* transcription reactions were performed on the same set of plasmid DNA topoisomers that were used in the experiments reported above. These transcription reactions were performed in the presence of a minimally saturating concentration of RNA polymerase, in the absence or presence of a minimally saturating concentration of Fis. Under these conditions, little Fis-mediated activation was observed on a relaxed DNA template, and activation increases with increasing negative DNA supercoiling, reaching a maximum of 2.8-fold at a superhelical density of $\sigma = -0.043$ (Fig. 5). Additionally, in the absence of Fis, transcriptional activity reached an optimum at a superhelical density of $\sigma = -0.069$. In the presence of Fis, this optimum decreased to $\sigma = -0.056$ (See Fig. 3) Thus, a supercoiled DNA template is required for Fis activation, and less global superhelical density is required for maximal transcriptional activity in the presence of Fis. This result supports the conclusion that additional local superhelical energy is delivered to the promoter site by Fis binding to the upstream SIDD region. That is, the superhelical stress experienced by the promoter is the sum of the global superhelical energy of the plasmid DNA template and the local superhelical energy delivered to the promoter site by Fis binding in the upstream portion of the SIDD region.

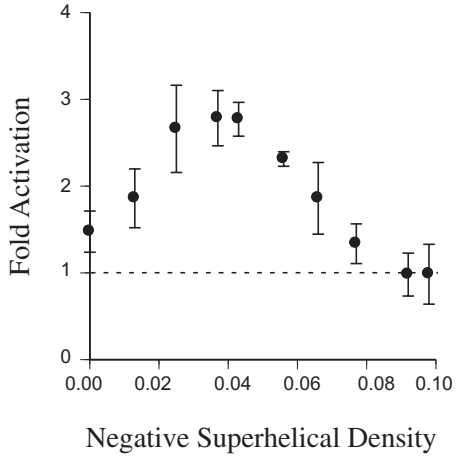


**Fig. 5.** Effect of negative DNA supercoiling on Fis-mediated activation from the *leuV* promoter. Values are *leuV* transcription rates in the presence of Fis divided by rates in the absence of Fis (obtained from the data presented in Fig. 3). Values are the mean ± SD of four separate experiments and are plotted against negative superhelical density. A dashed line represents no activation.

### *Fis increases the rate of open complex formation at the* leuV *promoter*

To test the prediction that Fis-mediated delivery of local superhelical energy from the upstream to the downstream portion of the SIDD domain increases the rate of RNA polymerase-mediated open complex formation at the *leuV* promoter, structural probing with $KMnO_4$ was used to measure rates of open complex formation in the presence or absence of Fis. These measurements were performed by preincubating a minimally saturating concentration of RNA polymerase, in the absence or presence of a minimally saturating concentration of Fis, with a plasmid DNA preparation at a mean superhelical density of $\sigma = -0.025$. Open complex formation was initiated with the addition of the first two initiating nucleotides (G and U), followed by $KMnO_4$ addition at 5, 10, 20, 30 and 40 s to visualize open complex formation. The results of this experiment revealed that Fis increases the rate of open complex formation 1.8-fold (Fig. 6).

### *Fis-mediated activation of open complex formation occurs in the absence of specific protein interactions between Fis and RNA polymerase*

If Fis activation of open complex formation occurs solely through the binding-induced transmission of superhelical energy from the upstream Fis site to the downstream *leuV* promoter, and is independent of the effects of specific contacts with RNA polymerase, then DNA binding by Fis that does not make such contacts would elicit the same response. To test this hypothesis, we needed a way of separating the effects of Fis-mediated activation through its specific interactions with the carboxyl-terminal domain of the $\alpha$-subunits of RNA polymerase from the DNA supercoiling-mediated activation described above. This was done using a mutant Fis protein, Fis R71K, that is incapable of making specific contacts with the $\alpha$-subunits of RNA polymerase, yet retains its wild-type DNA-binding and -bending properties (Bokal *et al*., 1997; McLeod

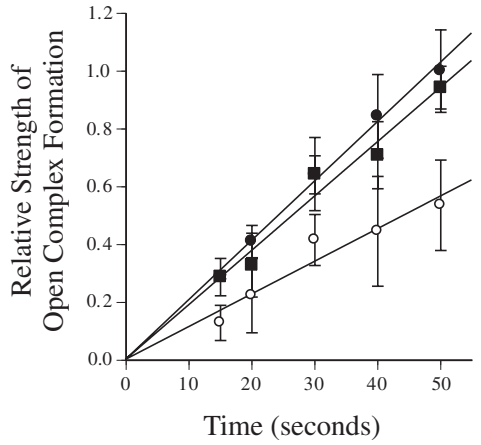


**Fig. 6.** Effect of Fis on open complex formation at the *leuV* promoter. Values are the strength of open complex formation divided by the strength of maximal open complex formation and are plotted against total incubation time as measured by the time between the addition of the first two initiating nucleotides and the termination of $KMnO_4$ probing. Maximal open complex formation was taken at times 110, 95 and 95 s, respectively, for samples in the absence of Fis (open circles), wild-type Fis (squares) and Fis R71K (filled circles). Open complex formation was measured as the sum of band intensities at positions −3, +2 and +5. Intraexperimental band intensities were normalized to each other relative to the sum of total counts incorporated in all major bands for each sample. Values are the mean ± SD of five separate experiments. $KMnO_4$ probing was performed as described in *Experimental procedures*.

*et al.*, 1999). $KMnO_4$ structural probing experiments were performed with the Fis R71K protein. The results given in Fig. 6 show that the mutant Fis R71K protein increases the rate of open complex formation at the *leuV* promoter as well as the wild-type Fis protein.

Although we were able to use this chemical probing method to demonstrate that the mutant Fis R71K protein can stimulate open complex formation, we were unable to demonstrate activation in a purified *in vitro* transcription system. Instead, transcription initiation is slightly inhibited (20%) by the mutant protein. One explanation is that the mutant Fis protein that cannot stabilize the closed RNA polymerase promoter complex limits access to the promoter by a scanning RNA polymerase.

### *Fis activates* leuV *transcription by multiple mechanisms*

Numerous reports have shown that Fis activates transcription at stable RNA promoters by making direct contacts with the carboxyl-terminal domain of the α-subunits or σ-subunit of RNA polymerase, which increase the RNA polymerase binding affinity (Zacharias *et al.*, 1991; 1992; Newlands *et al.*, 1992; Gosink *et al.*, 1993; Bokal *et al.*, 1995; 1997; McLeod *et al.*, 1999). Here, we show that Fis also activates transcription by a second mechanism involving a binding-induced, supercoiling-dependent transmission of destabilization from the upstream Fis binding site to the downstream promoter region. To determine whether these two modes of Fis-mediated activation occur independently of one another, RNA polymerase was titrated in the presence or absence of a saturating concentration of Fis using a plasmid DNA preparation with a mean superhelical density of $\sigma = -0.037$ as a DNA template for *in vitro* transcription reactions. This superhelical density was chosen because it produces maximal Fis-mediated activation in experiments conducted in the presence of a saturating concentration of RNA polymerase, 0.01 U $\mu l^{-1}$ (Fig. 5). At the lowest, subsaturating RNA polymerase concentration tested, Fis activated *leuV* transcription 4.5-fold (Fig. 7). However, as RNA polymerase was increased to a saturating concentration (.01 U $\mu l^{-1}$ and higher), Fis-mediated activation decreased to 2.5-fold. Because, at promoter-saturating polymerase concentrations, Fis can no longer activate transcription by polymerase recruitment, this residual activity must result from a DNA supercoiling-dependent step subsequent to RNA polymerase binding. The results presented above demonstrate that this subsequent step involves the facilitation of open complex formation.

## Discussion

While a large body of evidence demonstrates that Fis is a class I activator that enhances transcription by increasing RNA polymerase binding affinity through direct contacts with the carboxyl-terminal domain of the RNA polymerase α-subunits (Zacharias *et al.*, 1991; 1992; Newlands *et al.*, 1992; Gosink *et al.*, 1993; Bokal *et al.*, 1995; 1997; Zhi *al.*, 2003), others have suggested that

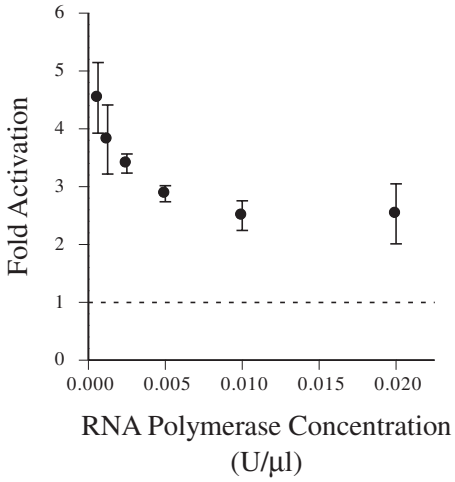


**Fig. 7.** Effect of RNA polymerase concentration on Fis-mediated activation from the *leuV* promoter. Values are *leuV* transcription rates in the presence of Fis divided by rates in the absence of Fis and are plotted against RNA polymerase concentration. Values are the mean ± SD of five separate experiments. A dashed line represents no activation.

additional mechanisms are involved in Fis-mediated activation at stable RNA promoters: Gourse and colleagues have suggested that Fis activates transcription from ribosomal RNA promoters by enhancing a step subsequent to closed complex formation (Bartlett *et al.*, 2000; Barker and Gourse, 2001); Sander *et al.* (1993) have shown that Fis increases the rate of promoter clearance at the ribosomal RNA *rrnD* promoter; and Muskhelishvili *et al.* (1997) have shown that Fis activates transcription from the promoter of the *tyrT* gene for tyrosyl-tRNA by enhancing the rates of open complex formation, promoter clearance and RNA polymerase binding affinity.

It is well established that DNA template topology changes (i.e. an imposed linking difference, $\Delta Lk$) in helicity, $\Delta Tw$, and/or DNA tertiary structure, $\Delta Wr$, can influence gene expression at all steps of bacterial transcription initiation (Hatfield and Benham, 2002; Muskhelishvili and Travers, 2003). It is also known that the global superhelical density of the bacterial nucleoid is homeostatically controlled by the competing activities of type I and type II topoisomerases. Therefore, if DNA template changes are to be used to modulate gene expression levels, mechanisms to control the superhelicity of short local DNA domains, independent of the global superhelicity of flanking DNA, must be used. Two mechanisms for this type of gene regulation have been proposed. One is based on protein-mediated changes in helicity, $\Delta Tw$, of the type proposed here for the *leuV* promoter (Sheridan *et al.*, 1998; Hatfield and Benham, 2002). The other is based on protein-mediated changes in tertiary structure, $\Delta Wr$, involving DNA microloops and microdomains proposed by Muskhelishvili and Travers (2003) for the regulation of tyrosyl-tRNA expression from the *tyrT* promoter of *E. coli*. In the *tyrT* system, Fis binds co-operatively to three helically phased sites upstream of the *tytT* gene and recruits RNA polymerase to the promoter. Of these three upstream sites, only the promoter-proximal site, located at basepair position −71, is required for RNA polymerase recruitment. Fis binding to the sites located further upstream stimulates both promoter opening and promoter clearance in a DNA supercoiling-dependent manner (Auner *et al.*, 2003). Muskhelishvili and Travers (2003) suggested that the DNA bending induced by Fis stabilizes backside contacts with RNA polymerase, thus creating a stable microloop with left-handed writhe. In this model, torsional strain is transferred to the DNA helix as the RNA polymerase aligns the suboptimally spaced (16 bp) −10 and −35 regions. It is also suggested that this torsional stress within the microdomain, defined by the Fis-stabilized RNA polymerase contacts with the DNA helix, facilitates strand separation and open complex formation.

These models involving protein-induced changes in either twist or writhe are not mutually exclusive, they are quite compatible. For example, although both *tyrT* and *leuV* promoters contain a Fis binding site at basepair position −71 that functions to recruit RNA polymerase, *leuV* does not contain additional, helically phased, upstream Fis binding sites. Thus, it is unlikely that Fis-stabilized writhe generates torsional stress at the *leuV* promoter as at the *tyrT* promoter. Nevertheless, backside contacts with RNA polymerase and contacts with UP sequences could serve this function. At the same time, it is interesting to note that the DNA sequence containing the *tyrT* promoter and the upstream Fis binding sites defines a strong SIDD site (Fig. 8). Thus, it is likely that, in addition to stabilizing writhe, Fis binding to the upstream *tyrT* sites also transmits negative twist to the promoter region, as suggested for the *leuV* system.

In this report, we describe evidence that expression of the *leuV* promoter is enhanced by a Fis-mediated translocation of superhelical energy mechanism similar to the IHF-mediated, DNA supercoiling-dependent mechanism described previously for the *ilv*$P_G$ promoter (Sheridan *et al.*, 1998; Hatfield and Benham, 2002). Theoretical calculations show that, at a mid-physiological superhelical density of $\sigma = -0.055$, the DNA duplex of the promoter-regulatory region of the *leuV* operon is predicted to be moderately destabilized at a SIDD site extending from basepair position −94 to +43 (Fig. 2). An interesting feature of the SIDD profile shown in Fig. 2 is the sharp peak of duplex stability at the G+C-rich discriminator region between positions +1 and −8 (Fig. 2). Others have shown that the discriminator found in all stable RNA promoters is responsible for open complex instability caused by the high energy of activation required for open complex for-

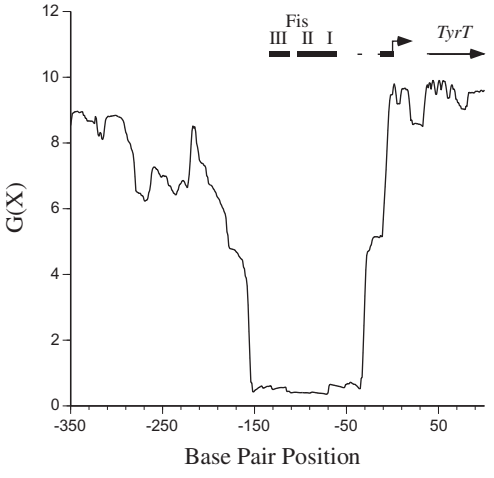


**Fig. 8.** SIDD profile of the *tyrT* promoter region from −350 to +100. Predicted free energies $G(x)$ for duplex destabilization at a superhelical density of $\sigma = -0.05$ at base position $x$ are expressed in kcal $mol^{-1}$ $bp^{-1}$. The positions of relevant promoter features are indicated above the SIDD plot. The transcriptional start site is indicated by an arrow. The −10 and −35 hexanucleotide regions are identified by thin horizontal lines. The discriminator is denoted by a thick horizontal line. The Fis binding sites are labelled.

mation (Gourse, 1988; Gaal *et al*., 1997; Pemberton *et al*., 2000). These findings explain the fact that *leuV* transcription is exceptionally sensitive to increasing levels of superhelical energy, increasing nearly 100-fold from its lowest level on a relaxed DNA template to its highest level on a supercoiled template (Fig. 3).

The SIDD profile shown in Fig. 2 predicted that the upstream region of the SIDD site might be stabilized in a B-form structure by Fis binding to a high-affinity target site centred at bp position −71. In this case, Fis could destabilize the downstream portion of this SIDD region and help to overcome the energy barrier posed by the discriminator to facilitate open complex formation at the *leuV* promoter site. These predictions were confirmed with *in vitro* transcription assays and $KMnO_4$ structural probing experiments performed with supercoiled DNA template topoisomers in the presence or absence of Fis and/or RNA polymerase (Figs 3 and 4). Additional experiments demonstrated that Fis binding enhances the rate of open complex formation and transcription initiation in a DNA supercoiling-dependent manner (Figs 5 and 6). Yet other experiments described here demonstrated that, at subsaturating concentrations of RNA polymerase, activation is facilitated by both Fis-mediated enhancement of RNA polymerase binding (Fig. 7) as well as DNA supercoiling-dependent enhancement of open complex formation (Figs 4 and 6). Evidence that this translocation of superhelical energy component occurs independently of protein interactions between Fis and RNA polymerase was provided by the observation that a mutant Fis protein, R71K (McLeod *et al*., 1999), which is defective for the formation of specific contacts with the α-subunits of RNA polymerase, but binds and bends DNA with wild-type properties, still promotes DNA supercoiling-dependent enhancement of open complex formation (Fig. 6). Even in the face of an increased rate of open complex formation, however, no mutant Fis-dependent transcriptional activation was observed. A plausible explanation for this might be that, in the presence of the mutant R71K Fis protein, a step subsequent to open complex formation, such as promoter clearance, becomes rate limiting. For example, it is likely that activation of transcription from the *leuV* promoter requires increases in negative twist in the promoter region contributed by both Fis-stabilized left-handed writhe as suggested by Muskhelishvili and Travers (2003) and Fis-mediated translocation of superhelical energy from the upstream SIDD region (Hatfield and Benham, 2002). As the mutant R71K Fis cannot form contacts with RNA polymerase to stabilize writhe to contribute negative twist, the remaining negative twist contributed by the SIDD mechanism might be enough to enhance the rate of open complex formation but not enough to increase the now rate-limiting step of promoter clearance.

In order to keep pace with rapid growth demands and high protein synthesis rates, stable RNA promoters have evolved to be among the strongest promoters in *E. coli*, accounting for 96% of total cellular RNA and 20% of total cell mass (Neidhardt and Umbarger, 1996). However, unchecked high rates of stable RNA synthesis during periods of limited growth and transitions from one nutritional or environmental state to another can deplete the cell of valuable energy. In response to these circumstances, stable RNA synthesis is subject to tight regulation. Although the mechanisms of these controls have been investigated extensively, they have not yet been completely elucidated (for reviews, see Condon *et al*., 1995; Cashel *et al*., 1996; Gourse *et al*., 1996; Keener and Nomura, 1996; Gaal *et al*., 1997; Bartlett *et al*., 2000; Barker and Gourse, 2001). The effects of Fis and DNA supercoiling on transcription from the *leuV* promoter provide additional information that might contribute to a more complete explanation of these mechanisms. Possible connections might involve the correlations between the control of chromosome superhelical density, energy charge and the growth state of the cell (Hatfield and Benham, 2002).

Fis levels jump from nearly undetectable in stationary phase to the most abundant DNA-binding protein in rapidly growing cells, but then decrease during the late log phase stage of growth (Ball *et al*., 1992; Nilsson *et al*., 1992; Azam *et al*., 1999). Likewise, the Fis binding sites upstream of the *rrnB* P1 promoter are near 100% bound during exponential growth in rich media, but Fis occupancy decreases in late log phase and becomes undetectable after continued incubation in stationary phase (Appleman *et al*., 1998). Steady-state levels of Fis vary with growth rate as does rRNA and most tRNAs, including LeuV (Ross *et al*., 1993). Here, we describe new regulatory properties of the *leuV* operon that suggest a way of co-ordinating modulations in negative DNA supercoiling and Fis protein levels with *leuV* transcription rates in response to changes in cellular energy charge reflected by nutritional and environmental conditions (Hatfield and Benham, 2002). Initiating nucleotide concentrations and ppGpp have also been documented to influence stable RNA synthesis in response to these conditions (Condon *et al*., 1995; Cashel *et al*., 1996; Gourse *et al*., 1996; Keener and Nomura, 1996; Gaal *et al*., 1997; Bartlett *et al*., 2000; Barker and Gourse, 2001). The fact that DNA supercoiling, Fis, NTP concentrations and ppGpp have all been proposed to mediate their effects through the stability of the promoter open complex and promoter clearance (Kajitani and Ishihama, 1984; Ohlsen and Gralla, 1992; Cashel *et al*., 1996; Bartlett *et al*., 2000; Barker and Gourse, 2001) suggests that all these factors might co-operate to achieve a maximal growth condition response at stable RNA promoters.

## Experimental procedures

### *Chemicals and reagents*

*Escherichia coli* RNA polymerase and restriction endonucleases were purchased from Roche Molecular Biochemicals. RNasin was purchased from Promega. [α-$^{32}$P]-UTP and [α-$^{32}$P]-dATP were purchased from Dupont/NEN. Nucleoside 5′-triphophates were purchased from Amersham Pharmacia Biotech. Deoxynucleoside 5′-triphosphates and the Klenow fragment of DNA polymerase I were purchased from New England Biolabs. Other chemicals were purchased from Sigma-Aldrich Chemicals.

### *Plasmids*

pKE-1 is a pBR322-based plasmid. The parent plasmid is pSL100 (Li *et al*., 1984). The *leuV* promoter region from −111 to +137 was joined with the *trp* attenuator termination region and inserted into the unique *Cla*I and *Hin*dIII sites of pSL100.

### *Generation of DNA plasmid topoisomers*

DNA plasmid topoisomers were generated essentially as described by Singleton and Wells (1982). Samples (10 μg) of plasmid DNA were incubated with 0–55 μM ethidium bromide and relaxed with 5 units of wheat germ topoisomerase I in a 40 μl reaction mixture containing 50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM NaCl and 10% glycerol for 3 h at 37°C. Each sample was extracted with phenol to remove ethidium bromide, extracted once with chloroform–isoamyl alcohol (24:1), precipitated with 100% ethanol, washed once in 70% ethanol and resuspended in 100 μl of water. Average linking number deficiencies were determined by separating a sample of each topoisomer preparation on several 1.4% agarose TAE (0.04 M Tris-acetate, 0.001 M EDTA) gels, containing ethidium bromide concentrations ranging from 0.04 to 2.0 μg ml$^{-1}$. Average linking number deficiencies were determined by the band counting method of Keller (1975). Average superhelical densities were calculated using the equation $\sigma = -10.5\Delta Lk/N$ (N = number of basepairs in the plasmid).

### In vitro *transcription reactions*

*In vitro* transcription reactions were performed essentially as described by Opel and Hatfield (2001). Aliquots of 50 ng (0.7 nM) of each DNA plasmid topoisomer preparation were preincubated in 20 μl of transcription buffer containing 10 μCi of [α-$^{32}$P]-UTP, 0.04 units μl$^{-1}$ RNasin, 0.05 M Tris-HCl (pH 8.0), 0.16 M NaCl, 5.0 mM $MgCl_2$, 0.1 mM DTT, 100 μg ml$^{-1}$ bovine serum albumin (BSA), 200 μM CTP, GTP, ATP and 20 μM UTP at room temperature. Where appropriate, preincubation reactions also contained 50 nM Fis. Unless otherwise noted, transcription reactions were initiated with the addition of a final concentration of 0.01 U μl$^{-1}$ RNA polymerase. Transcription reactions were terminated 2 and 4 min after transcription initiation by transferring 7 μl of sample to 7 μl of stop solution (95% formamide, 5 mM EDTA and 0.025% each bromophenol blue and xylene cyanol). Transcripts were separated by electrophoresis on a 6% denaturing polyacrylamide gel (5.7% acrylamide, 0.3% N,N′-methylene bisacrylamide) containing 8 M urea in a 0.09 M Tris-borate, 2 mM EDTA solution. Transcripts were quantified by phosphorimager analysis. Transcription rates were calculated where the production of transcripts versus time is linear and RNA polymerase is minimally saturating. Relative transcription rates were determined for each DNA topoisomer template preparation in relation to the topoisomer preparation showing the highest rate of transcription for each plasmid set. Relative transcription rates between different plasmid sets were normalized by comparison with the rate of RNA I transcription from the *ori* region of pBR322-based plasmids. For normalization of DNA topoisomer concentrations within a given set of topoisomers, DNA concentrations were determined by spectrophotometer analysis of absorbance at 260 nm.

### *$KMnO_4$ probing*

Aliquots of 200 ng (2.2 nM) of each DNA plasmid topoisomer preparation were preincubated in a 25 μl reaction containing 0.05 M Tris-HCl (pH 8.0), 0.16 M NaCl, 5.0 mM $MgCl_2$, 0.1 mM DTT and 100 μg ml$^{-1}$ BSA at 37°C. Preincubation reactions also contained 0.01 U μl$^{-1}$ RNA polymerase and/or 50 nM wild-type Fis or 200 nM Fis R71K. Reactions were initiated with the addition of a final concentration of 200 μM GTP and UTP and allowed to incubate for the indicated times. All reactions were probed at a final concentration of 20 mM $KMnO_4$ for 10 s at 37°C. Reactions were terminated with the addition of a final concentration of 572 mM 2-mercaptoethanol. Reactions were precipitated in ethanol and washed twice in 70% ethanol. The 3′ end of the coding strand at +62 relative to the transcription initiation site was labelled with [α-$^{32}$P]-dATP by digestion with *Nhe*I and end filled with DNA polymerase I Klenow fragment. The end-labelled fragment was subsequently released with *Bgl*II and isolated on a 6% polyacrylamide gel (5.7% acrylamide, 0.3% N,N′-methylene bisacrylamide) in a 0.09 M Tris-borate, 2 mM EDTA solution. The appropriate band was excised from the gel and eluted in 500 μl of AMES solution (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulphate). The eluted DNA was precipitated in ethanol and washed with 70% ethanol. Sites of $KMnO_4$ modification were detected by digestion with 30 μl of 10% piperidine for 30 min at 90°C. Piperidine was removed by evaporation at 90°C for 30 min. The DNA was resuspended in 20 μl of water and evaporated at 90°C for 20 min two times. DNA was resuspended in 10 μl (95% formamide, 5 mM EDTA and 0.025% each bromophenol blue and xylene cyanol). Transcripts were separated by electrophoresis on an 8% denaturing polyacrylamide gel (7.6% acrylamide, 0.4% N,N′-methylene bisacrylamide) containing 8 M urea in a 0.09 M Tris-borate, 2 mM EDTA solution. Band intensities were quantified by phosphorimager analysis.

### *SIDD plot analysis*

Theoretical analysis of the unwinding propensity of the experimental plasmid at various superhelix densities was performed using the technique developed previously by Benham

(1992). This method calculates the statistical mechanical equilibrium distribution of a population of identical, superhelical DNA molecules among all available states of denaturation. It then evaluates the equilibrium probability $p(x)$ of denaturation at single basepair resolution. It also calculates the destabilization energy $G(x)$, the incremental free energy required to ensure that the basepair at position $x$ is denatured. Thus, high values of $G(x)$ occur at positions where the duplex is relatively stable and low values at easily stress-destabilized sites. Positions where $G(x)$ is near zero are denatured with high probability at equilibrium under the assumed level of superhelicity. The calculation of $G(x)$ enables one to find fractionally destabilized sites – locations that have a low equilibrium probability of denaturation, but where other processes can drive duplex opening with a relatively small incremental input of energy.

All the free energy and conformational parameters used in these calculations have been measured experimentally, usually in multiple ways and under a variety of environmental conditions. So there are no free (i.e. tuneable) parameters in these analyses. Yet their results have been shown to be in quantitative agreement with experiment in all cases where experiments have been performed. They accurately predict both the locations and the extents of destabilization experienced by a DNA molecule of known base sequence, on which a specific level of superhelicity has been imposed (Hatfield and Benham, 2002).

It is important to note that DNA denaturation behaves differently when it is driven by superhelicity within topological domains than it does when driven by temperature in unconstrained molecules. In the latter case, only near neighbour effects are involved. However, the superhelical constraint couples together the behaviours of all basepairs within the domain involved. This coupling occurs because denaturation of any basepair alters its twist, which changes the partitioning of the superhelicity throughout the domain. In this way, every basepair is affected by the denaturation of any other basepair within the domain. This makes the transition behaviour of a domain potentially complex and interactive, with the opening of some regions coupled to the reversion back to B-form of others.

The calculations reported here were performed using the energy parameters appropriate for the nuclease digestion procedure of Kowalski *et al*. (1988), at a variety of superhelix densities as described.

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