Activation of Gene Expression by a Novel DNA Structural Transmission Mechanism That Requires Supercoiling-induced DNA Duplex Destabilization in an Upstream Activating Sequence*

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We have previously demonstrated that integration host factor (IHF)-mediated activation of transcription from the ilvPc promoter of Escherichia coli requires a supercoiled DNA template and occurs in the absence of specific interactions between IHF and RNA polymerase. In this report, we describe a novel, supercoiling-dependent, DNA structural transmission mechanism for this activation. We provide theoretical evidence for a supercoiling-induced DNA duplex destabilized (SIDD) structure in the A + T-rich, ilvPc regulatory region between base pair positions +1 and −160. We show that the region of this SIDD sequence immediately upstream of an IHF binding site centered at base pair position −92 is, in fact, destabilized by superhelical stress and that this duplex destabilization is inhibited by IHF binding. Thus, in the presence of IHF, the negative superhelical twist normally absorbed by this DNA structure in the promoter distal half of the SIDD sequence is transferred to the downstream portion of the SIDD sequence containing the ilvPc promoter site. This IHF-mediated translocation of superhelical energy facilitates duplex destabilization in the −10 region of the downstream ilvPc promoter and activates transcription by increasing the rate of open complex formation.

Negative superhelicity imposed on a DNA domain can drive local transitions to several types of alternate DNA conformations. These include cruciform extrusion at inverted repeat sequences, Z-DNA at alternating purine-pyrimidine sequences, and denaturation (also called local melting) that concentrates at A + T-rich sites (1–3). Each of these transitions requires free energy, and each releases free energy because the change of local helicity that occurs upon formation partially relaxes the imposed superhelicity. In a domain containing a single susceptible site, the transition will occur when the energy released exceeds the energy required to perform the transition (4).

In domains that contain multiple sites that are susceptible to transition, a complex competition among them will occur (1, 5). Whether a specific transition occurs depends not just on its local sequence but also on how well that transition competes with all others elsewhere in the domain. For example, David Lilley and colleagues (2) showed that the melting transition of an A + T-rich region contained within a supercoiled DNA plasmid required a significantly higher level of negative supercoiling when a (TG)12 region able to form left-handed Z-DNA under superhelical tension was inserted into the plasmid. This shows that the presence of a more susceptible site can inhibit less favorable transitions at other positions. In other words, superhelix-induced structures compete with one another for the negative superhelical energy required for their formation.

The transition behavior of superhelical domains also may be influenced by proteins that bind at or near these transition sites. For example, if a DNA region that is favored to form a superhelix-induced alternate structure is trapped in the B-form by the binding of a protein, the transition may be transferred to another region in the domain. If this second site is the −10 region of a promoter, this translocation of destabilization of the B-form can be used to activate transcription initiation by facilitating open promoter complex formation. In this report, we present evidence for this type of a DNA structural transmission mechanism for the regulation of gene expression in Escherichia coli.

Integration host factor (IHF) binds to an upstream activating sequence (UAS1) and activates transcription from the downstream ilvPc promoter of the ilvGMEDA operon of E. coli. This activation occurs in the absence of specific protein interactions between IHF and RNA polymerase, is not the consequence of a DNA looping mechanism, and requires a superhelical DNA template (6–8). IHF binding in the UAS1 region of a superhelical DNA template results in duplex destabilization in the −10 region of the downstream ilvPc promoter site. This DNA structural change at the downstream promoter site is correlated with an increase in the rate of open complex formation and a concomitant increase in the rate of transcription initiation (7). In this report, (i) we provide theoretical evidence for a supercoiling-induced DNA duplex destabilized (SIDD) structure in the A + T-rich, ilvPc regulatory promoter region between bp positions +1 and −162; (ii) we present experimental evidence to show that the local B-form DNA structure in the UAS1 region immediately upstream of the IHF binding site is destabilized by superhelical stress; (iii) we show that IHF binding prevents this duplex destabilization; (iv) we show that the threshold superhelical densities required for IHF activation and for the destabilization of this upstream portion of the SIDD region in UAS1 are similar; and (v) we demonstrate that the presence of this upstream destabilized structure is required for

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1 The abbreviations used are: IHF, integration host factor; UAS, upstream activating sequence; SIDD, supercoiling-induced duplex destabilization; bp, base pair(s); ΔLk, linking number difference; a, superhelical density.
IHF activation and IHF-mediated duplex destabilization in the –10 region of the downstream promoter. These observations suggest a novel, superhelically dependent DNA structural transmission mechanism of the type described above; i.e. binding of IHF prevents the superhelical destabilization of the upstream region but promotes duplex destabilization in the –10 region of the ilvP2 promoter at the other end of the SIDD structure. This IHF-mediated duplex destabilization in the –10 region of the ilvP2 promoter lowers the energy of activation for open complex formation and increases the rate of transcription initiation (7). This mechanism accounts for the facts that IHF activation of transcription from the ilvP2 promoter is DNA supercoiling-dependent and occurs in the absence of specific interactions with RNA polymerase.

**MATERIALS AND METHODS**

*Chemicals and Reagents—*Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs. *E. coli* RNA polymerase, pancreatic RNasin, and DNase I were purchased from Boehringer Mannheim. Radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech. DNA sequencing was performed using the Sequenase™ kit of U.S. Biochemical Corp. DNA oligonucleotides were purchased from MWG Biotech AG Inc. All other reagents were purchased from Sigma.

*Restriction Enzyme Analysis—*Restriction enzyme mapping was performed using the Sequenase™ kit of U.S. Biochemical Corp. DNA oligonucleotides were purchased from MWG Biotech AG Inc. DNA sequence analysis was performed using the Sequenase™ kit of U.S. Biochemical Corp. DNA was sequenced on an automated DNA sequencer using the Sequenase™ kit of U.S. Biochemical Corp. DNA was sequenced using the Sequenase™ kit of U.S. Biochemical Corp.

*DNase I Footprinting of IHF Binding to Wild Type and Mutant ilvP2 UAS1 Regions—*DNase I footprinting assays were performed to determine the location of IHF binding to the ilvP2 promoter-regulatory region of various constructs contained on a negatively supercoiled DNA template. Supercoiled plasmid DNA (1 × 10−9 M final concentration; σ = −0.06) was incubated for 20 min at 25 °C in the absence or presence of purified IHF (30 nM final concentration) in the same assay mixture used for DNase I digestion. The DNA was treated with 5 nM DNase I for exactly 2 min to ensure single-hit kinetic conditions (8). DNase I reactions were stopped by boiling the samples in boiling water for 5 min. The DNA was collected by precipitation with 2-propanol and resuspended in distilled water. Sites of DNase I-specific cleavage were visualized by the primer extension analysis described below with single-stranded DNA oligonucleotide primers that anneal to ilv DNA sequences 5’ (ilv bp positions −297 to −187) (9) of the UAS1 and ilvP2 promoter regions on the nonsense and sense strands, respectively.

*Mung Bean Nuclease Assays—*1.0 μg of supercoiled plasmid DNA template was digested with mung bean nuclease (0.1 units) in a 50-μl reaction (45 μM Tris borate (pH 7.6), 1 mM ZnCl2, and 1 mM EDTA) for 15 min at 25 °C. Free and IHF-bound DNA fragments were visualized by autoradiography following exposure of the samples to autoradiographic film (DuPont).

*Base Pair Resolution Mapping of Chemical Modification and Nuclease-specific Cleavages—*The sites of chemical modification and nuclease cleavage described above were mapped by primer extension using T7 DNA polymerase and ilv-specific oligonucleotides. After nuclease or chemical treatment, the DNA plasmid template was denatured in the presence of 200 mM NaOH for 10 min at 37 °C. 100 ng of an oligonucleotide primer was added, and the DNA was renatured with 300 mM NaOAc (pH 5.2). The renatured DNA was precipitated with isopropanol, washed once with 70% ethanol, and dried. The DNA pellet was resuspended in distilled water. The sites of KmI-DNA modification were resolved using primer extension mapping as described below with the single-stranded DNA oligonucleotide primers described above. The sites of IHF-DNA modification were resolved using primer extension mapping as described below with the single-stranded DNA oligonucleotide primers described above.

*Potassium Permanganate Probing Assays—*1.0 μg of supercoiled plasmid DNA was treated with 3 mM potassium permanganate in a 50-μl reaction (45 μM Tris borate (pH 7.6) and 1 mM EDTA) at 37 °C. After exactly 4 min, the reaction was stopped by the addition of 3 μl of β-mercaptoethanol. The modified DNA was precipitated with isopropanol alcohol, collected by centrifugation, washed twice with 70% ethanol, and dried. The DNA pellet was resuspended in distilled water. The sites of chemical modification and nuclease cleavage described above were mapped by primer extension using T7 DNA polymerase and ilv-specific oligonucleotides. After nuclease or chemical treatment, the DNA plasmid template was digested with mung bean nuclease (0.1 units) in a 50-μl reaction (45 μM Tris borate (pH 7.6) and 1 mM EDTA) for 15 min at 25 °C. Free and IHF-bound DNA fragments were visualized by autoradiography following exposure of the samples to autoradiographic film (DuPont).

*In Vitro Transcriptions—*The closed circular supercoiled plasmids pDH32wt and pSS298 (Table I) were used as DNA templates in vitro transcription assays performed in the absence and presence of purified IHF protein. RNA polymerase-plasmid DNA complexes were formed by preincubating 0.5 units (1.2 pmol) of RNA polymerase and 250 ng of plasmid DNA (0.1 pmol) in a 45-μl reaction mixture (0.04 M Tris-HCl (pH 8.0), 0.1 mM MgCl2, 0.1 mM dNTP, 0.1 mM EDTA, 0.025% each bromophenol blue and xylene cyanol). In order to determine the base pair location of modified and cleaved sites, primer extension for dideoxy sequencing was performed as described above with 1.0 μg of untreated plasmid DNA and the same ilv-specific oligonucleotide primers except for the addition of the appropriate dNTP (10 μM) to the initiation buffer. The primer extension products were separated by electrophoresis on an 8% denaturing polyacrylamide gel (7.6% acrylamide, 0.4% N,N'-methylenebisacrylamide) containing 8 μl urea in TBE buffer (90 mM Tris borate (pH 8.0), 1 mM EDTA) and visualized by autoradiography following exposure of the gels to Kodak XAR-5 film at −70 °C in the presence of a Cronex Quanta III intensifying screen (DuPont).

**Activation by DNA Duplex Destabilization**

Activation by DNA Duplex Destabilization

200 μM CTP, 20 μM UTP, 10 μCi of [α-32P]UTP (3,000 Ci/mmol), 100 μg/ml bovine serum albumin, and 40 units of RNasin) for 10 min at 25 °C. Transcription reactions were initiated by the addition of 5 μl of a 2 mM ATP, 2 mM GTP solution. Reactions were terminated after 2, 4, 6, 8, and 10 min by removing a 10-μl sample into 10 μl of stop solution (95% formamide, 0.025% xylene cyanol). The reaction products were separated by electrophoresis on an 8% denaturing polyacrylamide gel (7.6% acrylamide, 0.4% N,N'-methylenebisacrylamide) containing 8 m urea in TBE buffer and visualized by autoradiography following exposure of the gels to Kodak XAR-5 film at −70 °C in the presence of a Cronex Quant III intensifying screen (DuPont).

A 20-μl sample of each plasmid was treated with 20 units of Drosophila melanogaster topoisomerase II in a 40-μl reaction mixture (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, 1 mM ATP, and ethidium bromide (0–20 μm)) for 5 h. Each plasmid DNA sample was extracted three times with phenol to remove the ethidium bromide, precipitated with 2 volumes of isopropl alcohol, and resuspended in 100 μl of TE (10 mM Tris (pH 8.0) and 1 mM EDTA) containing 8 M urea in TBE buffer and visualized by autoradiography.

The results of these calculations are presented in Fig. 2, C and D. The SIDD profiles for the ilvGMEDA operon of E. coli. The SIDD profiles for the ilvFpo promoter region from bp −185 to +1 is shown. The nucleoid is numbered corresponding to the transcriptional start site from the ilvFpo promoter. The IEF core binding site, 5’-AAACAA-CATTATA-3’, in the upstream activating sequence UAS1 is located between bp −82 and −96 as indicated. The −10 and −35 hexamer nucleotide regions of the ilvFpo promoter are underlined. The SIDD region (−1 to −162) is identified by the dashed bracket, and the superhelicity destabilized region in the UAS1 (−98 to −153) is determined by OsO4 probing and identified by a bracket.

**RESULTS**

SIDD Is Predicted to Occur in the UAS1 Region at a Physiological Superhelical Density—The base pair composition of the ilvFpo promoter-regulatory region is exceptionally A + T-rich (Fig. 1). The 80-bp segment from bp positions −67 to −153 is approximately 88% A + T (9). In order to predict the stability of this region under superhelical stress, DNA SIDD profiles (1, 10) were calculated for plasmid pDHλwt. This plasmid contains the ilvFpo promoter region from bp position −248 to +6 (9) in a pBR322-based vector containing transcriptional terminators downstream of the ilvFpo start site (Table I). Calculations were performed using the energy parameters appropriate for the nuclease digestion procedure of Kowalski (1, 3). The results of these calculations are presented in Fig. 2, A and B. These are destabilization profiles. plots of the incremental free energy, G(x), required to guarantee denaturation of the base pair at position x. The smaller the value of G(x), the greater the destabilization experienced by that base pair. Values near 0 occur at sites where the B-form DNA base pair interactions are almost completely destabilized. The calculation profiles show that the DNA duplex in the UAS1 region is predicted to be destabilized in this plasmid at a physiological superhelical density of σ = −0.05. In fact, the predicted DNA destabilization of the UAS1 region in this plasmid context is even more pronounced than the well characterized melting transition of the A + T-rich sequence around bp position 3100 in the 3’-region of the β-lactamase gene (3). A close up view of the UAS1-ilvFpo promoter region of this SIDD profile is shown in Fig. 2C. These data show that the DNA duplex in the promoter region is predicted to be more stable than it is in the upstream UAS1 region.

Physical Evidence for Superhelically Induced Duplex Destabilization in UAS1 Region—The SIDD profile for pDHλwt (Fig. 2), predicts duplex destabilization in the ilvFpo promoter-regulatory region at a physiological superhelical density (σ = −0.05). This prediction was tested by chemically and enzymatically probing the DNA structure of this region in a set of pDHλwt DNA topoisomers, each prepared at a defined superhelical density. Each plasmid topoisomer was treated in the absence or presence of IEF with osmium tetroxide (OsO4), which modifies thymine residues in DNA regions where the

**FIG. 1.** Nucleotide sequence of the ilvFpo promoter-regulatory region of the ilvGMEDA operon of E. coli. The nucleotide sequence of the ilvFpo promoter region from bp −185 to +1 is shown. The nucleoid is numbered corresponding to the transcriptional start site from the ilvFpo promoter. The IEF core binding site, 5’-AAACAA-CATTATA-3’, in the upstream activating sequence UAS1 is located between bp −82 and −96 as indicated. The −10 and −35 hexamer nucleotide regions of the ilvFpo promoter are underlined. The SIDD region (−1 to −162) is identified by the dashed bracket, and the superhelicity destabilized region in the UAS1 (−98 to −153) is determined by OsO4 probing and identified by a bracket.

**FIG. 2.** Autoradiograph showing two-dimensional gel electrophoresis of plasmid DNA topoisomers. A 20-μl sample of each plasmid was treated with 20 units of Drosophila melanogaster topoisomerase II in a 40-μl reaction mixture (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, 1 mM ATP, and ethidium bromide (0–20 μm)) for 5 h. Each plasmid DNA sample was extracted three times with phenol to remove the ethidium bromide, precipitated with 2 volumes of isopropl alcohol, and resuspended in 100 μl of TE (10 mM Tris (pH 8.0) and 1 mM EDTA) containing 8 M urea in TBE buffer and visualized by autoradiography.

**FIG. 3.** Generation of predicted stress-induced duplex destabilization profiles. The transition properties of superhelical DNA molecules were calculated using a statistical mechanical analysis that evaluates the equilibrium properties of a population of identical, superhelically stressed DNA molecules in which every base pair is regarded as being susceptible to denaturation (1, 5, 10, 33). All states are examined individually, and the cumulative influence of the high energy states is estimated as a density of states calculation. In each state, the superhelical constraint is divided among three deformations, the change of helicity consequent to denaturing the collection of base pairs that are open in that state, helical interwinding of the two strands comprising the resulting denatured region(s), and the residual superhelicity that remains to stress the molecule. The free energies associated with these deformations have been determined by experimental observation. Co-polymeric denaturation energetics are assumed, in which the free energy of opening a base pair depends only on whether it is AT or GC. The other energy parameters are given the values that have been found to pertain under experimental conditions in which T = 37 °C, pH = 7.0, and the DNA is placed in a low ionic strength buffer containing 10 mM Tris-HCl and 1 mM Na2EDTA. Under these experimental conditions, comparison with experimental observations has shown the results of these calculations to be quantitatively accurate. We note that the pH and ionic conditions of the OsO4 and nuclease digestion experiments reported here are somewhat different that those assumed in the calculations.

Here the results of calculations are depicted as destabilization profiles. The free energy G(x) that is required to assure opening of the base pair at position x is calculated for each state as shown in the calculation. Low values of G(x) occur at positions where the base pair is substantially destabilized, whereas high values are found when the base pair is not destabilized by the imposed superhelical stresses. Destabilization profiles provide more information than plots of opening probability versus position, because they also show sites where the duplex is substantially destabilized but not sufficiently to cause denaturation with high probability. Such sites can be biologically important if another process or molecule can provide only an incremental amount of free energy to the opening reaction.
helix is in a form that exposes C-5–C-6 bonds (11) or with mung bean nuclease, which cleaves single-stranded DNA (3). OsO₄ reacts with destabilized sites, including premelted or “breathing” regions of A + T-rich DNA sequences, as well as with melted DNA. Nuclease digestion, in contrast, appears only to detect stably denatured regions. The locations of the chemically modified or enzyme-cleaved sites were determined by primer extension analysis as described under “Materials and Methods.”

In the absence of IHF, and at average negative superhelical densities between \( \sigma = -0.015 \) and \( -0.028 \) (Fig. 3, lanes 2 and 3), OsO₄ reactivity is observed at thymine residues located between bp positions \(-98 \) and \(-116 \). At average negative superhelical densities of \( \sigma = -0.450 \) and higher, OsO₄ reactivity spreads to bp position \(-116 \) (Fig. 3, lanes 4–8). Although primer extension detection of OsO₄ reactivity at the primer distal thymine residues is diminished at the higher superhelical densities, primer extension analysis from the other direction shows that the reactivity of all the thymines in this region on the other strand increases with increasing supercoiling.³ Thus, it is clear that the boundaries of the destabilized region in UAS1 are at bp positions \(-98 \) and \(-153 \).

While the results of the calculations presented in Fig. 2 predict superhelically induced duplex destabilization from bp position \(-70 \) to \(-156 \), the experimental results show that only the primer-distal portion of this sequence from bp positions \(-98 \) to \(-153 \) is, in fact, destabilized (Fig. 3). However, given the constraints of the theoretical calculations and the experimental conditions employed here, there is no reason to suppose that these results should be absolutely correlated. The SIDD analyses are based on experimental results obtained in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (3). However, in order to correlate the OsO₄ results with the mung bean nuclease digestion and two-dimensional gel electrophoresis analyses reported below, these experiments were performed in the buffer conditions required for electrophoresis (45 mM Tris borate, 0.5 mM EDTA, pH 7.0). Although such differences in ionic strengths between theory and experiments can affect some of the details of the transition, extensive calculations on many sequences have shown that predictions of the regions experiencing destabilization are quite robust, as are the qualitative differences in destabilization among multiple sites. Thus, the predictions of these attributes can be expected to apply under the experimental conditions reported here; but, predictions of the absolute amount of destabilization or the precise extent of denaturation have been shown to be accurate only under the conditions assumed in the calculations.

In order to determine whether or not the OsO₄ pattern is the result of stable unwinding of the DNA helix into a single-stranded form, we treated the plasmid pHΔwt with single-stranded DNA-specific mung bean nuclease. Unlike OsO₄, ³ S. D. Sheridan and G. W. Hatfield, unpublished data.
mung bean nuclease does not exhibit extensive reactivity in the UAS1 region. Single-stranded nuclease cleavage sites are observed only at base pair positions −112 and −113 (Fig. 3B). Thus, complete strand separation only occurs within the central portion of the UAS1 region that has been shown by OsO4 reactivity to be destabilized by superhelicity. Superhelical densities as high as \( \sigma = -0.10 \) do not denature the DNA helix throughout this region into a completely single-stranded state. The physical nature of this destabilized structure is currently unknown.

In order to determine the effect of IHF binding on superhelically induced destabilization of the DNA duplex in the UAS1 region, the OsO4 and mung bean nuclease structural probing experiments were repeated with the same supercoiled plasmid DNA templates in the presence of a saturating concentration (30 nM) of IHF. In the presence of IHF, neither OsO4 (Fig. 3C) nor mung bean nuclease3 showed any reactivity even at superhelical densities as high as \( \sigma = -0.10 \). Thus, IHF binding to its target site in the UAS1 region stabilizes the DNA duplex in the upstream UAS1 region, preventing the superhelically induced DNA duplex destabilization that occurs in its absence.

**Similar Superhelical Density Thresholds Are Required for IHF-mediated Activation and Superhelically Induced DNA Duplex Destabilization**—The superhelical density threshold for IHF-mediated activation of transcription from the ilvP_G promoter in pDHAwt occurs between superhelical densities of \( \sigma = -0.03 \) and \( -0.04 \) (8). If superhelically induced duplex destabilization in the UAS1 region and IHF-mediated activation are functionally correlated, then the threshold superhelical densities required for these two events should be similar. To test this prediction, we employed two-dimensional agarose gel electrophoresis. This method has the ability to accurately determine the threshold superhelical density required to initiate the formation of a superhelically dependent DNA secondary structure to a precision of \( \pm \Delta Lk \) of 1 or, in the case of plasmid pDHAw, \( \pm \sigma = 0.003 \) (12). For this measurement, a set of plasmid pDHAw topoisomers was created, pooled, and resolved by twodimensional electrophoresis as described under “Materials and Methods.” Fig. 4, A and B, shows that pDHAw exhibits a gradual, DNA superhelically dependent structural transition, beginning at a linking number of \( \Delta Lk = -15 \). No increase in writhes is observed from a linking number of \(-16 \) to the limit of resolution of the gel. This lack of change in writhes over a range of linking numbers is consistent with a gradual untwisting of the DNA helix. However, since the pBR322-based construct, pDHAw, contains other superhelically dependent structures (3), we could not be certain that the extension of this transition was due solely to the unwinding of the DNA helix in the UAS1 region. We therefore placed the ilvP_G promoter DNA region contained in pDHAw (ilv bp positions −248 to +6) into the unique BamHI site of pUC19 to create pSS19Awt (Table I). Since pUC19 does not contain any supercoiling-dependent DNA structures (Fig. 4C), only the DNA superhelically destabilized structure in UAS1 is detected in pSS19Aw (Fig. 4D). This UAS1-specific structural transition initiates at a threshold superhelical density of \( \sigma = -0.032 \). Thus, these experiments demonstrate the initiation of a DNA superhelically induced structural transition in the UAS1 sequence at a threshold superhelical density that correlates well with the superhelical density required for IHF-mediated activation (8).

**Deletion of the A → T-rich UAS1 Region Upstream of the IHF Binding Site Eliminates Superhelically Induced Duplex Destabilization in the UAS1 Region**—The data presented above demonstrate that the threshold superhelical densities required for IHF-mediated activation of transcription from the ilvP_G promoter and the DNA supercoiling-induced duplex destabilization in the UAS1 region are similar. This correlation suggests that destabilization of the DNA duplex in the UAS1 region might be required for IHF-mediated activation. In order to investigate the possibility that these events are functionally related, we created a deletion in which the superhelically destabilized DNA sequence in the UAS1 region was removed without altering the downstream IHF binding site. The UAS1 region upstream of the IHF site in this plasmid (pSS19-A; Table I) was replaced with vector DNA of an essentially random base composition (Fig. 5).

The results of mung bean nuclease and OsO4 structural probing experiments showed that the remaining, promoter-proximal half of the UAS1 region containing the IHF site is insensitive to modification in plasmid pSS19-A at superhelical densities throughout the range of \( \sigma = 0.00 \) to \(-0.07 \). This suggested that the region containing the IHF binding site upstream of the ilvP_G promoter in plasmid pSS19-A retains a double-stranded, B-form, DNA structure at superhelical densities that destabilize the full-length UAS1 region in plasmid pDHAw. This conclusion is consistent with the results of the two-dimensional gel electrophoresis experiment shown in Fig. 6.

Qualitatively, plasmid pSS19-A does not show the same DNA supercoiling-induced transition as plasmid pDHAw (compare Figs. 4B and 6B). pSS19-A exhibits a transition that initiates at a linking number deficiency of \(-14 \) (\( \sigma = -0.038 \)), which we assumed to be the well characterized, unstable region common to pDHAw and pSS19-A located around bp position 3100 in the SIDD plot shown in Fig. 2A (3). Alternatively, it was possible that the residual transition observed with pSS19-A occurred in the remaining portion of the UAS1 region. To confirm that this was not the case, we transferred the ilv-specific sequences in pSS19-A (ilv bp positions −98 to +6) into the unique BamHI site of pUC19 to create pSS19-A (Table I). The results of two-dimensional gel electrophoresis experiments show that plasmids pUC19 (Fig. 4C) and pSS19-A (Fig. 6A) do not exhibit any DNA supercoiling-induced structural transitions throughout the superhelical density range assayed (\( \sigma = 0.01 \) to \(-0.06 \)). We conclude, therefore, that the gradual DNA superhelically induced DNA duplex destabilization observed in pSS19-Awt is ilv-UAS1-specific and that this DNA structural transition requires the promoter-distal half of the UAS1 region.

**IHF Binds to Its Target Site in the UAS1 Region of pSS19**—Binding sites for IHF consist of a core consensus sequence, WATCAANNNTTR (where W represents A or T, N represents any base, and R represents pyrimidine), and frequently contain a flanking dA-dT element located immediately upstream of this core sequence (13, 14). Although the functional role of this dA-dT element is unclear, it has been suggested to influence the binding of IHF through indirect contacts with the minor groove of the DNA helix (13, 15). Rice et al. (15) have recently analyzed the structure of a co-crystal with the H9 site of phage λ DNA and IHF. They showed that the interaction of IHF with an A tract on the 5′-side of the core sequence is stabilized by the presence of a narrow minor groove created by the poly(dA-dT) sequence and suggested that this interaction is facilitated by structural rather than by sequence specificity. The IHF binding site in the UAS1 region also contains a dA-dT sequence (5′-TATTATTTTAAAAA-3′) on the 5′-side of its core binding sequence. While this sequence is disrupted in pSS19-A, an upstream 5′-AATAAAA-3′ sequence is retained (Fig. 5B). In order to ensure that we had not disrupted the ability for IHF to bind to its core binding site in the UAS1 region, gel mobility shift assays were performed, and the affinities of IHF binding to its target site in the UAS1 region of plasmids pDHAw and pSS19-A were compared (Fig. 7). The \( K_a \) value for IHF binding
IHF binds to the same site on both plasmids. Thus, the deletion of the sequences upstream of the IHF binding site does not eliminate the binding of IHF to its target binding site. We have previously shown that IHF also binds with nearly identical affinities to relaxed and supercoiled DNA templates (8).

Deletion of the A + T-rich UAS1 Region Upstream of the IHF Binding Site Eliminates IHF-mediated Superhelically Induced Duplex Destabilization in the −10 Region of the ilvP₂ Promoter—The results of the experiments described above demonstrate that the superhelically destabilized structure in UAS1 is required for IHF-mediated activation. Our proposed mechanism suggests that the IHF-mediated inhibition of the formation of this structure activates transcription by shifting some of the superhelical stress normally absorbed by this structure in the absence of IHF to the downstream ilvP₂ promoter portion of the SIDD region described above (Fig. 1). If this is the case, then the binding of IHF to plasmid pSSA-98, which does not contain the upstream superhelically destabilized structure, should not destabilize the DNA duplex in the −10 region of the downstream ilvP₂ promoter. This prediction was tested by chemically probing the DNA structure in the −10 region of the ilvP₂ promoter with KMN₅O₄ and OsO₄ in the presence and absence of IHF on supercoiled plasmid pH₄wt and pSSA-98 DNA templates. The results shown in Fig. 8A were obtained by primer extension using an oligonucleotide that anneals to ilv bp positions −160 to −132 on the transcribed DNA strand. In the presence of IHF, KMnO₄ sensitivity is observed at thymines located on the transcribed strand at bp positions −11 and −12 in the promoter region (Fig. 8A, lane 1). In the absence of IHF, no KMnO₄ sensitivity is observed in this region (Fig. 8A, lane 2). IHF-induced duplex destabilization at these same nucleotide positions is detected with the more sensitive structural probing reagent OsO₄ (Fig. 8A, lane 3). Again, no destabilization is observed in the absence of IHF (Fig. 8A, lane 4). Since the upstream primer binding site is not present in pSSA-98, a DNA oligonucleotide that binds to a downstream sequence at bp positions 32–62 relative to the ilvP₂ transcriptional start site on the nontranscribed strand was used for chemical probing the DNA structure in the −10 region of the SIDD region described above (Fig. 1). If this is the case, the absence of IHF to the downstream region of the ilvP₂ promoter portion

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FIG. 3. Effect of increasing negative superhelical density on chemical and enzymatic reactivity of the ilvP₂ promoter region on topoisomers of plasmid pH₄wt in the presence and absence of IHF. A and C, topoisomers of plasmid pH₄wt were treated with 2 mM OsO₄ and 2 mM 2,2-bipyridine, and the sites of modification were mapped by ilvP₂-specific bp locations by primer extension as described under “Materials and Methods.” Average negative superhelical densities (μ) for A, lanes 1–9 (ΔLk = 0 to −40), are σ = 0.00, −0.058, −0.058, −0.058, −0.058, −0.070, −0.085, −0.10, and −0.10. Primer extension analysis in A, lane 9, was performed on the same topoisomer sample as lane 8 except that it was not treated with OsO₄. Base pair positions for all primer extension analyses were determined by dideoxy sequencing using the same oligonucleotide primer (lanes G, A, T, and C). The arrows indicate thymine residues (which correspond to adenines in the other strand in the sequencing ladder) modified by OsO₄. Average negative superhelical densities (μ) for B and C, lanes 1–9 (ΔLk = 0 to −40), are σ = 0.00, −0.015, −0.028, −0.045, −0.058, −0.070, −0.085, −0.10, and −0.10. As indicated above each panel, topoisomers of plasmid pH₄wt were treated with osmium tetroxide in the absence of IHF except for those presented in C, which contained a saturating concentration (30 mM) of IHF. B, topoisomers of plasmid pH₄wt were treated with mung bean nuclease in the absence of IHF, and the sites of nuclease-specific cleavage were mapped to ilvP₂-specific bp locations by primer extension. Additional experiments showing OsO₄ modification elsewhere in the plasmid have been performed using a different primer to ensure that the presence of IHF did not squelch the OsO₄ reaction.

FIG. 4. Two-dimensional agarose gel electrophoresis of topoisomers of plasmids pH₄wt, pUC19, and pSS19wt. Plasmid topoisomers of plasmids pH₄wt (A and B), pUC19 (B), and pSS19wt (D) were analyzed by two-dimensional agarose gel electrophoresis on a 21-cm 1.4% agarose gel. The first dimension (top to bottom) was run at 37 °C in 0.5× TBE at 70 V for 26 h. The gels were soaked in 1× TAE buffer containing 0.02 μg/ml ethidium bromide (A) or 0.1 μg/ml (B–D). The second dimension (from left to right) was performed in this same buffer at 25 °C until fully resolved. Tracings in A–D are presented for added clarity. The arrows indicate threshold linking number deficiency (and superhelical density (μ)) for structural transitions.
primer extension through the promoter region from the other direction. On this strand, IHF-induced sensitivity on pDH \( \text{D} \) \( \text{wt} \) (\( s = 0.06 \)) is observed at all thymines located between bp positions 17 and 218 (Fig. 8A, lane 1). However, in the absence of IHF (Fig. 8B, lane 2) or with pSSA-98 in the presence (Fig. 8B, lane 3) or absence of IHF (Fig. 8B, lane 4), OsO\(_4\) reactivity at these thymine sites is greatly diminished. These results provide strong evidence that IHF inhibition of duplex destabilization in UAS1 is responsible for IHF activation of duplex destabilization in the ilvPG promoter region.

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**FIG. 5. Construction of the pSSA-98 plasmid and deletion of the UAS1 5'-region.** A, diagrams of the plasmids pDH\( \text{D} \) \( \text{wt} \) and pSSA-98 used in this study. B, nucleotide sequence of the UAS1 region of the ilvPG promoter in these plasmids indicating the location of the IHF core binding site (underlined in boldface type) and the nucleotide sequence of the vector DNA (underlined) that was used to replace the A+T-rich wild type UAS1 5'-region.

**FIG. 6. Two-dimensional agarose gel electrophoresis of topoisomers of plasmids pSS19-98 and pSSA-98.** Topoisomers of plasmids pSS19-98 (A) and pSSA-98 (B) were analyzed by two-dimensional agarose gel electrophoresis on 21-cm 1.4% agarose gels. The first dimension (top to bottom) was run at 37°C in 0.5X TBE at 70 V for 28 h. The gels were soaked in 1X TAE buffer containing 0.1 mg/ml ethidium bromide for 6 h. The second dimension (from left to right) was performed in this same buffer at 25°C until fully resolved. Schematic diagrams for A and B are presented. The arrows indicate threshold linking number deficiency, (\( \Delta Lk \)) (and superhelical density (\( \sigma \))) for structural transitions.

**FIG. 7. Quantitative analysis of IHF binding to its target site in the ilvPG promoter region in pDH\( \text{D} \) \( \text{wt} \) and pSSA-98.** Shown are autoradiograms of gel mobility shift assays performed with a \( \gamma^32P\)-end-labeled, 471-bp EcoRI–BamHI DNA fragment from plasmid pDH\( \text{D} \) \( \text{wt} \) (containing ilvbp from 250 to +6) (top) or a 269-bp BstYI–BamHI DNA fragment from plasmid pSSA-98 (containing ilvbp from 98 to +6) (bottom) in the absence (lane 1) and increasing concentrations of IHF protein (lanes 2–8) as described under “Materials and Methods.” IHF concentrations in the binding reactions corresponding to lanes 2–8 are, respectively, 0.47, 0.94, 1.9, 3.8, 7.5, 15, and 30 nM. DNA concentrations used in these experiments were less than 1X10^-11 M.
In previous reports, we have shown that IHF binding in UAS1 causes the formation of a nucleoprotein structure that facilitates the destabilization of the DNA duplex in the −10 region of the ilvPG promoter and increases the rate of the isomerization step of the transcription initiation reaction (7). We have also shown that (i) in the absence of IHF, promoter activity increases with increasing DNA supercoiling; (ii) in the presence of IHF, the effect of DNA supercoiling on basal level promoter activity is amplified 5-fold; (iii) IHF binding does not alter the superhelical density of the DNA template; (iv) in the presence or absence of IHF, the relative transcriptional activities of the promoter remain the same at any given superhelical density; (v) IHF activation cannot be replaced by simply increasing the negative superhelical density of the DNA template; and (vi) IHF binds to relaxed and negatively supercoiled DNA templates with nearly identical affinities (8). These results demonstrated that although IHF activation requires a supercoiled DNA template, activation by DNA supercoiling and IHF are effected by separate mechanisms. Therefore, to accommodate these observations and because IHF-mediated activation was shown to occur in the absence of protein interactions between IHF and RNA polymerase, we considered the possibility of activation by a DNA structural transmission mechanism (7, 8).

The experimental results described in this report demonstrate a superhelically induced destabilization of the DNA helix in UAS1. IHF binding is shown to prevent these DNA structural changes. Furthermore, destabilization of this region in the absence of IHF binding is shown to occur at a similar superhelical density threshold required for IHF-mediated activation of transcription from the downstream ilvPG promoter (8). These correlations suggest a DNA structural transmission mechanism for this activation that involves DNA secondary structural transitions (Fig. 10). According to this mechanism, IHF binding in the UAS1 region of a superhelical DNA template inhibits the formation of an untwisted, superhelically induced, alternate DNA structure in which the B-form of the duplex is locally destabilized. In the absence of IHF, this structure is formed under negative superhelicity. In the presence of IHF, the formation of this structure is inhibited. Thus, the superhelicity normally absorbed by this structure in the absence of IHF must be accommodated by transitions at other DNA sites in the presence of IHF. Our experimental results demonstrate that one of these sites is located in the −10 region of the downstream ilvPG promoter where duplex destabilization facilitates open complex formation to activate transcription initiation. This mechanism is consistent with our previous demonstration that IHF binding alters the distribution but not the absolute level of the linking deficiency of a superhelical domain containing the UAS1 region. The experimental evidence reported here in support of this DNA structural transmission mechanism is discussed below.

Theoretical analysis of superhelically induced DNA duplex destabilization (Fig. 2) predicts strong destabilization in the A + T-rich promoter-regulatory region at physiologically relevant superhelical densities. Chemical and enzymatic probing of the structure of the DNA helix demonstrated that duplex destabilization initiates at a low, physiological, superhelical density around bp positions −98 and −116 in the UAS1 region of this region and spreads about 50 bp to bp position −153 as the negative superhelical density of the DNA template becomes more extreme (Fig. 3A). These experiments also showed that although the DNA helix in this region is destabilized, it is not entirely stably unwound (Fig. 3B). It is interesting that this superhelically destabilized region is confined to the promoter-distal half of the UAS1 region located immediately upstream of the IHF binding site. It does not extend into the IHF binding site or the farther downstream ilvPG promoter region (Fig. 1). In the presence of IHF, however, no duplex destabilization in the UAS1 region was observed (Fig. 3C). In this case, the superhelical deformation absorbed in the absence of IHF by destabilizing this region must be redistributed to other sites. Therefore, since IHF binding has been shown previously to cause DNA superhelicity-dependent duplex destabilization in the −10 region of the downstream promoter (7), it was plausi-
that this downstream destabilization was caused by the IHF-induced inhibition of transitions in the upstream UAS1 region. If this hypothesis is correct, then no activation would be expected in the absence of this structure. Since the region that experiences this destabilization is upstream from the IHF binding site in UAS1 (Figs. 1 and 3), it was possible to replace it with a superhelically stable DNA sequence without affecting the DNA sequence of the IHF binding and promoter sites (Fig. 5) or the ability of IHF to bind to its target site in the UAS1 region (Fig. 8). When this superhelically destabilized DNA sequence in UAS1 was replaced with a superhelically stable one, it was observed that, as expected, IHF could not activate transcription initiation from the \( \text{ilvPG} \) promoter, even on a highly supercoiled DNA template (Fig. 9). Furthermore, in the absence of the superhelically destabilized structure in UAS1, IHF binding to a supercoiled DNA template does not destabilize the DNA duplex in the \(-10\) region of the downstream \( \text{ilvPG} \) promoter (Fig. 7). These results demonstrate that IHF-mediated inhibition of the formation of this structure is responsible for the downstream duplex destabilization that leads to activation of transcription initiation from the \( \text{ilvPG} \) promoter.

This DNA structural transmission mechanism further predicts that the threshold superhelical densities required for duplex destabilization in the UAS1 region and for IHF activation of transcription initiation from the \( \text{ilvPG} \) promoter should be similar. No transmission would be expected at less extreme superhelical stress levels than those required for duplex destabilization in UAS1. Two-dimensional gel electrophoresis of topoisomer sets of the pDH\( \text{D} \)wt plasmid containing the \( \text{ilvPG} \) promoter-regulatory region suggest that this is the case. Negative superhelical densities of \( \sigma > -0.032 \pm 0.003 \) are required for duplex destabilization in the UAS1 region of this plasmid (Figs. 4 and 6). This value agrees well with our previously

**Fig. 9.** *In vitro* transcriptional analysis of \( \text{ilvPG} \) promoter activity in the absence and presence of IHF on supercoiled plasmids pDH\( \text{D} \)wt and pSS\( \Delta \)-98 (\( \sigma = -0.07 \)). *A*, *in vitro* transcriptions were performed in the presence (+IHF; 30 nM) or absence (−IHF) of IHF with supercoiled plasmid DNA templates of superhelical density \( \sigma = -0.07 \) as described under “Materials and Methods.” Transcription reactions were terminated after 2, 4, 6, 8, and 10 min, and the 157-nucleotide \( \text{ilvP}_1 \) transcript was isolated by electrophoresis on a denaturing 8% polyacrylamide gel containing 8 M urea and visualized by autoradiography. The 229-nucleotide \( \text{ilvP}_1 \) transcript arises from a fortuitous *in vitro* promoter in the A + T-rich UAS1 region present in the plasmid pDH\( \text{D} \)wt but not in plasmid pSS\( \Delta \)-98. Transcription from this promoter is repressed in the presence of IHF. The doublet transcription product bands are presumed to arise from heterogeneous termination. *B*, data obtained from the above *in vitro* transcription reactions were plotted, and the rate of transcript formation was determined by the slope of \( \text{ilvPG} \)-specific band intensity versus time in minutes. Fold activation was determined by dividing the rate of transcription in the presence of IHF by the transcription rate in the absence of IHF.

**Fig. 10.** DNA structural transmission (competing structural transitions) mechanism of DNA superhelix-dependent transcriptional activation of the \( \text{ilvPG} \) promoter by IHF. See text for discussion.
estimated threshold negative superhelical density of \( \sigma > -0.035 \pm 0.005 \) for IHF activation (8). It should be emphasized, however, that these values are not expected to be identical. This is because the conditions required for each of these measurements are different. The two-dimensional gel experiments were performed at a lower ionic strength (45 mM Tris borate) than the transcription reactions (100 mM KCl). Stable unwinding of A + T-rich regions is known to be suppressed at ionic strengths greater than 50 mM NaCl (16). Thus, a negative superhelical density more extreme than the threshold value measured by the two-dimensional electrophoresis experiment is expected in a transcription reaction that occurs at a higher salt concentration. On the other hand, destabilization of the UAS1 region at a less extreme threshold is predicted by the fact that additional negative supercoiling is generated in this region during basal level transcription from the downstream ilvP\(_2\) promoter (17–19). For example, Rahmouni and Wells (20) have shown the formation of a supercoiling-dependent B-DNA to Z-DNA transition upstream of an active promoter, although the average superhelical density present \textit{in vivo} should not have been high enough to induce this structural transition (19).

It has been documented that small sequence changes can radically alter the transition behavior of a supercoiled molecule if they change the relative competitiveness of different regions (5). Two examples of this behavior are relevant to this report. First, deleting a portion (even a small portion) of a strongly destabilized site can radically alter its competitiveness, causing the transition to shift to another location although most of the original site remains intact. In the case described here, removal of part of the UAS1 region destroys the ability of the remaining downstream promoter portion to become destabilized by superhelicity. Second, it is possible to alter transition behavior by constraining a portion of an easily destabilized DNA region to remain in the B-form. This also can have the effect of altering the competitiveness of the region involved sufficiently to shift the transition to another part of the same destabilized region or to another easily destabilized site. This is the essence of the mechanism proposed in this report; \textit{i.e.} IHF binding forces the superhelically destabilized site in the UAS1 region to remain in the B-form, which shifts part of the transition energy to the promoter site of this SIDD region and the rest to other easily destabilized sites.

Another IHF-mediated, supercoiling-dependent model for the regulation of transcription has been reported. Higgins \textit{et al.} (34) have suggested that IHF activates transcription from the phage \( \mu \) P\(_2\) promoter by forcing the promoter site to the outside of a superhelical node where it is in an ideal location for interaction with RNA polymerase. In support of this model, Van Rijn \textit{et al.} (35) demonstrated that this activation is dependent on the face of the helix and correlated with increased RNA polymerase binding affinity. In contrast, the IHF-mediated activation of transcription from the ilvP\(_1\) promoter is independent of the face of the helix and affects the kinetic step of open complex formation (7). Thus, a super-loop type model can be excluded for IHF-mediated regulation of transcription from the ilvP\(_2\) promoter. It was also noted that both IHF binding in the UAS1 region and the pSSA-98 construct eliminate \textit{in vitro} transcription from the ilvP\(_1\) promoter (Fig. 9). This suggested the possibility that IHF might act to facilitate downstream promoter selection by repressing transcription from the upstream promoter. However, previously published data demonstrate that this is also not the case (7). Point mutations in the UAS1 region that abolish transcription from ilvP\(_2\) but do not affect IHF-binding do not activate transcription from the downstream ilvP\(_1\) promoter. Furthermore, these point mutations do not affect IHF-mediated activation.

It is of interest to note that DNA sequences having higher than average A + T content are observed upstream of several strong \( \sigma^0 \) promoters and that alterations in these upstream A + T-rich regions affect the rates of transcription initiation from downstream promoters (21, 22). Since a considerable proportion of the negative supercoiling in the bacterial nucleoid is unconstrained, these A + T-rich regions can serve as local sites that are susceptible to destabilization. The results reported here demonstrate that regulatory proteins that bind to these upstream regions can affect the susceptibility of sequences to become destabilized by imposed superhelicity and transfer this susceptibility to nearby promoter sites. Therefore, the mechanism of protein-mediated, superhelicity-dependent, regulation of transcription initiation in prokaryotes that is reported here may be of general significance. For example, Fyfe and Davies (36) have recently reported that the deletion of an A + T-rich DNA sequence upstream of an IHF binding in the regulatory region of the \textit{Neisseria gonorrhoeae} pilE\(_1\) promoter, which has a striking resemblance to the ilvP\(_2\) promoter-regulatory region described here, eliminates IHF-mediated activation in this system. However, the DNA supercoiling dependence of this activation has not yet been examined.

Recent reports indicate that DNA superhelicity and superhelically destabilized structures also are important for the regulation of transcription in eukaryotes. For example, Emerson and colleagues (23) have shown that supercoiled DNA templates are required for enhancer-mediated \textit{in vitro} regulation of the chicken \( \beta^2\)-globin and mouse T-cell receptor genes. Also, Michelotti \textit{et al.} (24) have shown that the basal level of transcription from the c-myc gene generates negative DNA superhelicity that destabilizes upstream A + T sequences. These destabilized regions are targets for single-stranded DNA-binding proteins that are required for enhanced \textit{in vivo} transcription from this promoter. Thus, it appears that protein-stabilized DNA duplex destabilization might serve a range of roles in \textit{in vivo} regulatory mechanisms in both prokaryotes and eukaryotes.

In conclusion, it should be stressed that it is essential to separate the strands of the DNA duplex for many biological processes such as DNA replication, recombination, and transcription initiation in both prokaryotes and eukaryotes (25–27). Negative superhelicity lowers the energy of activation for strand separation. The data presented in this report demonstrate a level of regulation for these processes that involves a competition between potential destabilized sites that is mediated by protein binding. Therefore, since it is now apparent that DNA supercoiling is an integral component of many mechanisms involved in the regulation of cell growth and metabolism, a further elucidation of these types of protein-mediated, DNA supercoiling-dependent effects should enhance our understanding of a large number of regulatory systems.

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