Transcription-generated DNA supercoiling plays a decisive role in a promoter relay mechanism for the coordinated expression of genes in the *Salmonella typhimurium* *ileI*-leuO-leuABCD gene cluster. A similar mechanism also operates to control expression of the genes in the *Escherichia coli* *ileI*-leuO-leuABCD gene cluster. However, the mechanism underlying the DNA supercoiling effect remained elusive. A bacterial gene silencer AT8 was found to be important for the repression state of the *leuO* gene as part of the promoter relay mechanism. In this communication, we demonstrated that the gene silencer AT8 is a nucleation site for recruiting histone-like nucleoid structuring protein to form a cis-spreading nucleoprotein filament that is responsible for silencing of the *leuO* gene. With a DNA geometric similarity rather than a DNA sequence specificity, the *E. coli* gene silencer EAT6 was capable of replacing the histone-like nucleoid structuring protein nucleation function of the *S. typhimurium* gene silencer AT8 for the *leuO* gene silencing. The interchangeability between DNA geometrical elements for supporting the silencing activity in the region is consistent with a previous finding that a neighboring transcription activity determines the outcome of the gene silencing activity. The geometric requirement, which was revealed for this silencing activity, explains the decisive role of transcription-generated DNA supercoiling found in the promoter relay mechanism.

DNA supercoiling has been known to play important roles in transcriptional regulation (1–7). By using a bacterial transcription regulation model system, we have demonstrated that transcription-generated DNA supercoiling is a crucial driven force that triggers the sequential activation of genes in the *Salmonella typhimurium* *ileI*-leuO-leuABCD gene cluster (8–12). This rather complex sequential gene activation process was named the promoter relay mechanism (11, 12). The exact molecular detail that underlies the effect of transcription-generated DNA supercoiling on the sequential activation of genes at this locus remains unclear. The direct DNA supercoiling effect on activating promoters of genes in this region has been ruled out. Instead, the effect appears to mediate through cis-elements within the locus control regions (LCRs1 illustrated in Fig. 1) located between genes in the *ileI*-leuO-leuABCD gene cluster (8).

Although not ruling out the possible involvement of other cis-acting elements in the transcription regulation, we have identified two cis-elements in the LCR-1 that are important for the promoter relay mechanism as follows: a bacterial gene silencer, termed AT8; and a LeuO protein-binding site, termed AT7 (13, 14). The bacterial gene silencer AT8-mediated transcriptional silencing is integral to the gene expression regulation and is responsible for the repressed state of the *leuO* gene. LeuO protein-mediated derepression, which relieves the repression of *leuO* gene, is also a crucial part of the promoter relay mechanism. Transcription-generated DNA supercoiling is likely to play its roles in the promoter relay mechanism via modulating the processes of the repression-derepression control (10). To better understand the DNA supercoiling effect, we investigated the basic molecular criteria of the repression element (gene silencer) and derepression element (LeuO-binding site). We revealed that it is the geometric features of the DNA rather than the specific sequences of the transcription elements that are important for their transcription regulatory functions. The striking DNA geometric requirement is consistent with the involvement of transcription-generated DNA supercoiling in the transcription regulatory process.

The revealed DNA geometric features of the gene silencer also provided clues for the possible involvement in the transcription regulation of chromosome architectural proteins (*e.g.* HU, H-NS, Lap, and IFS, etc.) that usually recognize DNA structure rather than specific DNA sequence for their bindings (15–17). Indeed, a genetic screening has led to the identification of a histone-like nucleoid structuring protein (H-NS) for its role in the gene silencing mechanism. Functionally, we demonstrated that the gene silencer AT8 is indeed an H-NS nucleation site that triggers the formation of a nucleoprotein filament structure in the region. With the assistance of the neighboring AT-rich DNA in LCR-I, the transcriptional repressive nucleoprotein structure reaches (cis-spreads) to the promoter region of the *leuO* gene and results in the repression of the gene. Despite the low DNA sequence homology, the *Escherichia coli* gene silencer EAT6, but not a same size neutral DNA sequence, was capable of replacing the *S. typhimurium* gene silencer AT8 for its H-NS nucleation function in *S. typhimurium* LCR-I. Therefore, it is clear that regardless of the low DNA sequence specificity, either the *S. typhimurium* gene silencer AT8 or the *E. coli* gene silencer must provide the crucial DNA geometry for triggering the H-NS nucleation. The recruited H-NS, along with other nucleoproteins, appear to form...
a cis-spreading nucleoprotein filament that represses the activities of promoters located within the proximity.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Bacterial Strains**—Plasmid constructs: pAO, PEV101, and pWU204 have been described previously (12, 18). A 75-bp DNA (positions +48 to -27 of ilvIH), including the -10 sequence of the promoter of ilvIH, was deleted from pWU204 and resulted in pWU205; otherwise pWU205 is identical to pWU204.

The 393-bp *E. coli* LCR-1 was generated using PCR. Primers 5'-GTCACAACCTGACCTCATAAACAGCCTC-3' and 5'-GAATGATGATCTTACACTGTCATAAAATCTAAATG-3' were used in the PCR. The primers contain mismatches (the underlined sequences) for producing AatII restriction sites on both ends of the PCR product. The *E. coli* LCR-1 was inserted into the unique AatII site on pAO. Other pAO-based testing plasmids were also derived by using similar strategies, and those DNA inserts involved are individually described in the experiments.

The plasmid, pWU802, was derived from pWU804 (8) by deleting a 1.4-kb BamHI-NsiI fragment that includes the coding region of leuO gene and the downstream piloH. To replace AT8 DNA in *S. typhimurium*, the plasmid pWU902OZ was derived from pWU802 with EAT6 DNA, the following two DNA oligomers were chemically synthesized: 5'-taatataatataaatataaaagacatattatATCACTACTTCTG-3' and 5'-GTTAGATGATgattaaatgtgcttt-3', the lowercase DNA sequence is EAT6 DNA. The annealed DNA was used to replace the 58-bp HpaI-DraIII fragment containing AT8 in *S. typhimurium* LCR-1 on pWU802. With a similar approach, the following two synthetic DNA oligomers, consisting of DNA sequences flanking the footprinting reaction sites of the nucleotide sequence (part of the downstream coding region of *lacZ*), were used to replace AT8 on pWU802: 5'-aaccatacgtagcaagcaatctggctgctgacaatgacACTACATCTGTG-3' and 5'-GTTAGATGATgattaaatgtgctt-3'; the lower case is the DNA sequence used. The procedures resulted in precisely the replacement of the AT8 DNA sequence with either the *E. coli* repressor element EAT6 or the neutral DNA sequence to maintain the footprint DNA sequence intact. For *E. coli* pWU802-LA and pWU802-LH were derived from pWU802. DNA oligomers containing the 27-bp lac repressor binding sequence (lac operator) 5'-CGGAGATCTGACGGATACAAATTTTATGATNCG-3' and the flanking restriction sites were synthesized, annealed, and ligated at the AatII site or the HindIII site on pWU802 to generate pWU802-LA or pWU802-LH, respectively.

Plasmid pWU902OZ was derived from pWU802. The *leu-500* mutation (A to G transition) at the -10 region of the promoter of the *leu-ABCD* operon was converted back to the wild type -10 DNA sequence, otherwise the LCR-1 DNA, which controls the expression of the downstream leuO promoter, stayed the same as that in pWU802. The entire coding sequence of *lacZ* gene, along with its upstream Shine-Delgarno sequence, was positioned downstream of the transcription-initiation site of the *leuO* gene. This construction was used to express the *lacZ* gene product as a reporter for *leuO* promoter activity.

Plasmid pCH5015-(ATS)2 was derived from pCH301-(ATS)5, which has been described in a previous study (14). The coding region of the *sacB* gene was used to replace the coding region of gfpUV of pCH301-(ATS)5. The coding region of the *sacB* gene was amplified using PCR in the presence of DNA template pEX100T (19), which was kindly provided by Dr. Sylvia Rimsky, IGR, Villejuif Cedex, France) were incubated at room temperature for 30 min to a protein-DNA binding equilibrium. After the incubation, 1 ml of MgCl2 and 0.5 ml CaCl2 was added into each reaction for continued incubation at room temperature for 1 min. DNase I (units used per reaction is indicated in each figure legend) was then added to the reactions for a 90-s incubation at room temperature. The DNase I footprinting reactions were stopped by adding 140 μl of stop solution consisting of 192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, and 64 μg/ml yeast RNA. The samples were then phenol-extracted, ethanol-precipitated, and resuspended in gel loading buffer. To mark precisely the positions of the protein-mediated DNase I protection sites, primers used for PCR were individually used in the DNA sequencing reactions (Sequenase sequencing kit, U. S. Biochemical Corp.) for preparing DNA sequence ladders. By using a chemical cleavage reaction (24), a G + A marker was also prepared from the radioactively end-labeled DNA. Along with one of the position markers (the DNA sequence ladders or the G + A marker), the DNA products prepared from the footprinting reactions were analyzed on 7% acrylamide, 7 M urea denaturing PAGE.

**Tn5 Transposon Insertion-Mediated Random Mutagenesis and the Reverse Screening Procedure**—1 μl of EZ:TN752K (KAN-2) Tnp Transposon (27) (Epicerin), which contains the Tn5 transposon and the expression of the bacterial gfpUV gene, was mixed in a 0.5 ml of electrocompetent *E. coli* DH5α cells harboring pCH5015-(ATS)5 that were prepared by serial washes with cold 10% glycerol in the mid-exponential growth phase (A600 = 0.6). Electroporation was performed in a Bio-Rad Gene Pulser apparatus (set up at 25 microfarads, 200 ohms, and 2.50 kV). After adding 1 ml of SOC medium, the components were 20 g of bacitraputyn, 5 g of yeast extract, and 0.5 g of NaCl, pH 7.5, in 1 liter of H2O final concentrations of 20.
mm MgSO$_4$ and 20 mM glucose were added into the medium after autoclaving), the electroporated cells were transferred to a culture tube and incubated at 37 °C for 1 h. After incubation, cells were plated on LB agar plates containing 50 μg/ml ampicillin and 25 μg/ml kanamycin. Once colonies formed, they were replicated onto plates with or without 5% sucrose supplement.

β-Galactosidase Assay—The β-galactosidase assay was measured by hydrolysis of o-nitrophenyl β-galactosidase to produce o-nitrophenol in permeabilized bacterial cells as described previously (25).

Direct Genomic DNA Sequencing—Chromosome DNAs were isolated from the selected Tn5 knock-out strain and used for direct genomic DNA sequencing. Primers that hybridize to the ends of the inserted transposon, Tn5, were used for a bi-directional outward sequencing that reads into the genomic DNA, flanking the Tn5 insert. The obtained DNA sequences were used to pin-point the insertion site on the bacterial genome using the Blast search (NCBI Data Bank). The direct genomic DNA sequencing was performed at the facility of Fidelity Systems Inc. (Gaithersburg, MD).

RESULTS

Genes in the E. coli ilvIH-leuO-leuABCD Gene Cluster Are Regulated via the Promoter Relay Mechanism—The promoter relay mechanism was found based on the coordinated expression of genes in the S. typhimurium ilvIH-leuO-leuABCD gene cluster (12). By using a plasmid-borne S. typhimurium DNA context in E. coli hosts, we initially demonstrated that trans-acting protein factors important for the promoter relay mechanism are functionally available in E. coli strains. This is based on the observed activation of a plasmid-borne leu-500 promoter, the hallmark of the promoter relay mechanism, in the heterogeneous (E. coli protein factors acting on S. typhimurium DNA context) assay system (data not shown). The result of this initial test prompted us to directly monitor the mRNAs of ilvIH and leuO in MC4100, an E. coli relA1 strain under an experimental condition that triggers a severe starvation for branched-chain amino acids (b-caa), isoleucine, leucine, and valine, during the log phase of bacterial growth. The leuO gene in E. coli relA1 strain is normally silent and activated in response to the starvation for b-caa during exponential growth in 17-amino acid SSA, a synthetic medium supplemented with all amino acids except the three b-caa (18). The b-caa starvation causes a 2-h growth arrest (the slow-down period between points 2 and 3 shown in the growth curve in Fig. 1) prior to the growth resumption (the growth rate increase after point 3 in the growth curve shown in Fig. 1). We have demonstrated that the LeuO protein is required during the growth stress for cells to resume their growth after the 2-h growth arrest (growth stress) because the leuO knock-out strain, MFI, failed to resume its growth after the arrest (18).

According to the promoter relay mechanism, the production of LeuO in MC4100 cells during growth stress is presumably because of the activation of the leuO gene triggered by the transcription activity of ilvIH promoter. The ilvIH transcription activity-dependent leuO gene activation has been demonstrated previously in S. typhimurium cells that are entering the stationary phase (25); however, this has not been directly demonstrated in E. coli strains. Hence, we monitored the mRNAs of ilvIH and leuO during the growth of MC4100 in 17-amino acid SSA medium. Indeed, both ilvIH and leuO mRNAs were only detectable at the end of the 2-h growth arrest prior to the cell growth resumption (Fig. 1, B and C, lanes 3). Both mRNAs were not detected at the time points during the exponential growth (Fig. 1, B and C, lanes 1), and at the beginning of the 2-h growth arrest (Fig. 1, B and C, lanes 2). This Northern result indicated that the ilvIH operon is indeed activated during growth stress (2-h growth arrest). The transcriptional activity of the ilvIH operon is expected to subsequently activate the leuO gene via the promoter relay mechanism (11, 12).

The co-detection of ilvIH and leuO mRNAs during the 2-h growth arrest can best be explained by the promoter relay mechanism. Therefore, we expected that the deletion of ilvIH promoter activity would also abolish the activity of the leuO...
gene under the same experimental condition (MC4100 cells under the starvation for b-caa). Hence, the ilvIH transcription activity-dependent activation of leuO was tested using a pair of multicopy plasmids, pWU204 and pWU205, that both carry the E. coli promoter relay DNA sequence (NCBI GenBank/EBI data bank accession number AF106955). These two plasmids are identical except that the ilvIH promoter (−10 sequences of ilvIH operon) has been deleted from the E. coli promoter relay DNA sequence on pWU205, whereas the promoter of the ilvIH operon on pWU204 remains intact (Fig. 1). The plasmids were tested in MC4100 cells grown in 17-amino acid SSA for the activity shown at the promoter end of the regulatory region, because both EAT1 and EAT3 did not show any significant repression states of the leuO gene as an integral part of the promoter relay mechanism. Because the promoter relay mechanism is also responsible for the transcriptional regulation of the genes in the E. coli ilvIH-leuO-leuABCD gene cluster (14), the two elements were located in the locus control region I (LCR-I) upstream of the divergently arrayed leuO and leuABCD (illustrated in Fig. 1). These two elements are responsible for the repression and the derepression states of the leuO gene on pAO. This was, however, consistent with a previous observation that the DNA sequence in the region (LCR-I) is AT-rich but otherwise shares little DNA sequence homology between the two closely related enteric bacteria (26). Because it was impossible to identify the cognate transcription elements in E. coli LCR-I, based on the known DNA sequences of S. typhimurium transcription elements, we searched for transcription elements in E. coli LCR-I based on the known properties of the transcription elements. E. coli Repression Element—Based on previously established criteria (13, 14), we assayed for gene silencing activity (repression element) in the 393-bp E. coli LCR-I (the AT-rich DNA located between the −20 position and the −412 position of the E. coli leuO gene) flanked by leuO and leuABCD (illustrated in Fig. 2). The results showed that gene silencing activity is located near the leuO promoter end of the regulatory region, because both EAT1 and EAT3 did not show any significant gene silencing activity in the assay (Fig. 2, lanes 3 and 5). The gene silencing activity was finally narrowed down to EAT6, a 39-bp AT-rich DNA sequence located between −84 and −122 positions of the leuO gene. This location is consistent with the

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**FIG. 2. The identification of a repression element in E. coli LCR-I.** The E. coli LCR-I (a 393-bp DNA segment that consists of DNA sequences between the −412 and −20 position of the E. coli leuO gene) was divided into several segments as illustrated. Each DNA segment was individually inserted at the AatII site, which is located 95 bp upstream of the transcription initiation site (+1) of the β-lactamase gene (bla) on pAO. Primer extension was used to monitor the activity of pbla for searching the transcriptional repression activity in the E. coli LCR-I. The quantified pbla activity shown at the bottom of each lane for three repeated experiments is expressed as the mean within the range of ±0.004 (S.D.).
possibility that the *E. coli* repression element EAT6 is also directly responsible for the repression state of the *E. coli* *leuO* gene in a manner analogous to that of the *S. typhimurium* repression element AT8, in control of the *S. typhimurium* ilvIH-leuO-leuABCD gene cluster (14).

**E. coli Derepression Element**—Again, by using our previously established criteria (14), we searched for the derepression element (LeuO-binding site) in the region between the transcription start site (+1 position) of the *E. coli* *leuO* gene and the repression element EAT6 (illustrated in Fig. 3). Based on the results of EMSA, by tracing down the binding region, a LeuO-binding site, 29-bp EAT16, was identified at a position downstream of the *E. coli* repression element EAT6 and upstream of the transcription initiation site of the *E. coli* *leuO* gene (Fig. 3). A DNase I footprinting experiment (Fig. 4) revealed that the LeuO protein-dependent DNase I protection was indeed initiated at the LeuO-binding site EAT16 (+38 to −66 position of *E. coli* *leuO* gene) in LCR-I (Fig. 4A, lane 2). Upon the increase of LeuO protein concentration, the LeuO-mediated DNase I protection was extended toward the regions (zones 1 and 2) flanking EAT16 (Fig. 4A, lanes 3 and 4). Although the LeuO-mediated DNase I protection in the center region of EAT16 is less clear on the complementary strand of the LCR-I DNA (Fig. 4B), a similar DNase I protection in zones 1 and 2 was observed on the complementary strand in the presence of a high concentration of LeuO protein (Fig. 4A, lane 1). Whether the extension of LeuO protein-mediated DNase I protection beyond the LeuO-binding site EAT16 at a very high protein/DNA ratio has any biological significance in the transcription regulation, remains to be tested in the future. Nonetheless, the DNase I footprinting result (Fig. 4) and the EMSA data (Fig. 3) together supported that LeuO binding in *E. coli* LCR-I was initiated at the LeuO-binding site EAT16. This is consistent with the relative positions of the repression element, derepression element, and the targeting *leuO* promoter found in the *S. typhimurium ilvIH-leuO-leuABCD* gene cluster (14). Hence, a similar repression-derepression process may indeed be responsible for the repression and the transient activation (derepression) of the *E. coli* *leuO* gene as an indispensable part of the promoter relay mechanism in the *E. coli* ilvIH-leuO-leuABCD gene cluster (10).

**Functional Replacement of the DNA Sequence Heterogeneous *E. coli* and *S. typhimurium* Elements in the Repression-Derepression Process**

FIG. 3. Identification of a LeuO-binding site located between *E. coli* repression element and the promoter of the *E. coli* *leuO*. DNA oligomers consisting of the DNA sequences of the various DNA segments in the region (as illustrated) were chemically synthesized and annealed for interacting with an increasing amount of purified histidine-tagged LeuO protein in EMSA. The results of EMSA are summarized along with the list of the name of each DNA segment. The + sign indicates a positive result of the LeuO binding assay for the DNA segment. The − sign indicates a negative result of the LeuO binding assay for the DNA segment. The EMSA results of several key DNA segments are shown.
Gene silencers, intrinsic DNA curvatures were predicted using required for retaining the transcriptional regulatory functions. activities exerted by the elements because all the identified limited homologous regions do not explain the transcription the cognate sequences (Fig. 5).

The similarity observed was that both repression elements are extremely AT-rich (about 60% AT). The best possible DNA sequence homology between the two repression elements is 31% (Fig. 5A). The similarity observed was that both E. coli repression and derepression elements are functionally interchangeable for the transcription-derepression process despite the DNA sequence heterogeneity.

The E. coli Repression Element Is Functional for Regulation of leuO Expression in the Context of S. typhimurium LCR-I—In prior experiments, the activities of the repression-derepression elements were assayed with a reconstituted plasmid DNA context. To confirm the functional compatibility of the transcription elements in a native environment (the DNA context of S. typhimurium LCR-I), because the replacement of the promoter of the leuO gene (pLeuO) by using primer extension (Fig. 6A). As expected, the S. typhimurium repression element AT8 is indeed responsible for the repression of the leuO gene in its native context (S. typhimurium LCR-I), because the replacement of the S. typhimurium repression element AT8 with a same-size neutral DNA abolished the repression of pLeuO activity (Fig. 6A, lane 3). Most strikingly, the transcriptional repression was restored when the E. coli repression element EAT6 was used to replace the S. typhimurium repression element AT8 (Fig. 6A, lane 2). This is a strong piece of evidence that the E. coli repression element exerts a reasonable transcriptional repression activity in context of the S. typhimurium ilvIH-leuO-leuABCD gene cluster, despite the lack of apparent DNA sequence homology between the E. coli and S. typhimurium LCR-I. The gene silencing effect appears to extend some distance because the promoter of the β-lactamase gene (pbla), which is located 392 bp downstream, was also affected when either the S. typhimurium repression element AT8 or the E. coli repression element EAT6 was present (Fig. 6B).

The results therefore showed that both repression and derepression elements were functionally interchangeable for the control of the expression of genes in the ilvIH-leuO-leuABCD gene cluster. Instead of DNA sequence homology, DNA geometrical similarities may be important for the transcription regulatory functions of the repression and derepression elements.
This complex mechanism involving DNA geometrical changes stabilized by transcription-generated DNA supercoiling is likely to be responsible for the repression-derepression process. If so, this is a novel transcription regulatory mechanism that deserves further investigation to define the underlying molecular details. As the first step, we focused on the repression mechanism. Although the cis-acting repression element (gene silencer) has been well characterized thus far, the trans-acting protein factors responsible for gene silencing remain unknown.

A Genetic Approach for the Identification of Genes Required for Bacterial Gene Silencing—A two-step screening procedure involving an initial reverse selection (as described under “Experimental Procedures”) followed by a positive screen was used to identify gene(s) important for the bacterial gene silencing mediated by the gene silencer AT8. *Bacillus subtilis* sac*B* gene encodes levansucrase, which catalyzes the hydrolysis of sucrose resulting in the synthesis of levans (29). In the presence of 5% sucrose, expression of sac*B* in Gram-negative bacteria such as *E. coli* is lethal (30). The *E. coli* harboring “suicide” plasmid, pCH501S-(AT8)*2*, carrying the coding region of the sac*B* gene under the control of the *leuO* promoter, which is repressed by the direct repeat of the bacterial gene silencer AT8, was used in the first step screening for genes that are important for the gene silencing mechanism. AT8 dimer (AT8)*2* provided a very tight gene silencing effect as demonstrated in our previous study (14). Because the promoter of the *leuO* gene is one of the natural promoters regulated by the gene silencer, expression of the lethal gene sac*B* on the suicide plasmid is strongly repressed in the presence of (AT8)*2*. To screen for genes required for silencing, Tn5 transposon insertion mutagenesis (as described under “Experimental Procedures”) was used to randomly knock-out genes throughout the bacterial genome. If a Tn5 insertion knocked out a gene important for the gene silencing mechanism, *E. coli* harboring the suicide plasmid would no longer survive on LB plates containing 5% sucrose because of the relief of gene silencing. However, that mutant
can still form a colony on LB without sucrose. By using this reverse selection procedure, we obtained 398 clones out of a total 2,926 colonies from this negative screening. Because Tn5 transposon may also target plasmid DNAs, we excluded the clones containing plasmids that carry the Tn5 inserts from the 398 clones. This exclusion resulted in 300 potential positive clones selected from the first-step reverse selection procedure.

Because the reporter gene sacB is very toxic for Gram-negative bacteria used in the reverse selection, we expected many of these 300 selected clones might contain knock-out genes important for sucrose metabolism or sugar transport, rather than genes directly relevant to AT8-mediated gene silencing. A preliminary titration experiment to determine the appropriate concentration of sucrose (titration range, 10 to 0.5% sucrose in LB) to be used for the initial reverse selection procedure had indicated that the sucrose toxicity is very stringent. Whereas 5% sucrose was chosen as a toxicity threshold in the initial reverse selection, we noticed that even a small leakage of the activity of the promoter that controls the expression of sacB could result in toxicity in the presence of as little as 0.5% sucrose. Hence, the 300 potential positive clones were grown in LB supplemented with 0.5% sucrose as a second-step screening procedure. Most strikingly, we found that with the exception of one clone, the rest of the 299 clones survived in the culture of LB supplemented with 0.5% sucrose. Although it is possible that some of the 299 excluded clones may be genes that play minor roles in the silencing mechanism, the single positive clone isolated in the second-step screening procedure must contain a knock-out gene crucial for the bacterial gene silencing. This was confirmed using pWU9020Z to report the gene silencing activity in the isolated Tn5 knock-out clone. Plasmid pWU9020Z carries the entire regulatory region (LCR-I) of the S. typhimurium leuO gene and the leuO promoter that controls the expression of the downstream coding sequence of lacZ reporter gene. Because of the effect of the gene silencer located in the LCR-I region, the expression of the reporter lacZ gene is repressed. If the Tn5 insertion had knocked out a gene that was truly important for the silencing activity in the LCR-I, then we expected an increase of the expression of the reporter lacZ gene. Indeed, compared with pWU9020Z harboring DH5α (the parental strain of the Tn5 knock-out strain), a 16-fold increase of β-galactosidase activity was found in the isolated Tn5 knock-out strain harboring pWU9020Z (data not shown).

**H-NS Is the Trans-acting Factor Responsible for the Transcriptional Repression Mediated by the Gene Silencers**—Direct genomic DNA sequencing was used to identify the site of Tn5 insertion on the chromosome of the positive clone isolated. The DNA sequencing result indicated that the Tn5 insertion is located at the +59 position of the coding region of the hns gene. The insertion is likely to cause either early termination or truncation of the translation product of the gene because it is in
the N terminus of the coding region. Hence, H-NS, the gene product of hns, is most likely to be the protein factor important for the gene silencing mechanism.

The possibility was further confirmed in experiments using a pair of isogenic hns'/hns− strains (Fig. 7). Two testing plasmids were used in the experiments. First, two testing plasmids, pAO with or without the gene silencer sequence (AT4), which had been used to characterize the bacterial gene silencer in our previous studies (13, 14), were used to confirm the involvement of H-NS in the bacterial gene silencing (Fig. 7A). The results showed that a reduced gene silencing activity was observed when pAO-AT4 was tested in the hns− strain (Fig. 7A, lane 4) compared with the silencing activity detected in the hns+ strain (Fig. 7A, lane 2). The 4-fold reduction on the gene silencer AT4-mediated gene silencing must be due to the absence of H-NS in the hns− strain because the genetic background of the two bacterial strains is identical except for the hns gene. In a second experimental set, we directly monitored the effects of the gene silencer on the promoter of the leuO gene on the testing plasmid pWU802 series (Fig. 6). The presence of either the S. typhimurium silencer AT8 or the E. coli silencer EAT6 in the S. typhimurium LCR-I was able to repress the promoter activity of the leuO gene on the pWU802 plasmid series (Fig. 7B, lanes 1 and 2). The gene silencing effect was clearly H-NS-dependent because the repression of leuO promoter activity was significantly reduced in the hns− strain (Fig. 7B, lanes 4 and 5). Moreover, the repressive effect of H-NS on the promoter activity of leuO gene must be mediating through either the S. typhimurium silencer AT8 or the E. coli silencer EAT6, because the replacement of the gene silencer with a neutral sequence failed to affect the promoter activity of leuO gene in either testing condition (Fig. 7B, lanes 3 and 6). The gene silencing effect on the relatively distal bla promoter was also H-NS-dependent because a 2-fold reduction on the gene silencing effect was found in the hns− strain (Fig. 7B, compare lanes 7 and 8 with lanes 10 and 11). The repression activity assay was carried out in the absence of the interference of LeuO protein because the leuO gene was knocked out in the isogenic pair of hns+/hns− strains used in the experiment.

The Gene Silencer Important for the Promoter Relay Mechanism Is an H-NS Nucleation Site—Because the H-NS-dependent gene silencing activity is absolutely dependent on the presence of either the S. typhimurium gene silencer AT8 or the E. coli gene silencer EAT6 in LCR-I, H-NS must affect the promoter activity with a biological event specifically initiated at the gene silencer. The DNA sequence required for triggering the initiation process of H-NS-mediated gene silencing has been termed the H-NS nucleation site (31, 32). Based on this definition, an H-NS nucleation site is distinct from other ordinary H-NS-preferred binding DNA sequences. H-NS nucleation is absolutely required for the formation of a well organized nucleoprotein structure, which is shown to be transcriptionally repressive. Other ordinary H-NS-binding DNA sequences may lead to the binding of H-NS, but in the absence of an H-NS nucleation site, the bound H-NS is not necessarily well organized and hence is not transcriptionally repressive (31).

DNase I footprinting assays were used to investigate whether the identified gene silencer (AT8 or EAT6) is in fact an H-NS nucleation site. The results showed that the S. typhimurium gene silencer AT8 was the first region to be protected by H-NS in the DNase I footprinting experiment (Fig. 8, left panel, lane 3). With increasing H-NS concentration in the DNase I footprinting experiment (Fig. 8, left panel, lanes 2 and 1), the protected region gradually extended toward the promoter of the leuO gene. A detailed titration of H-NS concentration was carried out to better demonstrate the gradual extension of the H-NS-dependent protection in the DNase I footprinting experiment (Fig. 8, right panel). Clearly, the S. typhimurium gene silencer AT8 is the site occupied by H-NS as the first step. Although the DNase I protection was also slightly extended toward the 5′ direction (Fig. 8, right panel, lanes 1–4), the 3′ end extension was much more significant (Fig. 8, right panel, lanes 1–4), and the DNase I protection eventually reached the promoter of the leuO gene (Fig. 8, left panel, lanes 1–3). Hence, this DNase I footprinting result demonstrated that the S. typhimurium gene silencer AT8 is indeed an H-NS nucleation site, which initiates the formation of a transcriptionally repressive nucleoprotein structure. This structure has a potential to extend (cis-spread) toward either direction; however, the remaining AT-rich DNA sequence in LCR-I may confer directionality to the cis-spread nucleoprotein structure. In this case, the transcriptionally repressive nucleoprotein structure preferentially extended toward the promoter of the leuO gene.

Similar H-NS nucleation and unidirectional cis-spread were also observed when E. coli LCR-I was tested in a similar DNase I footprinting experiment (Fig. 9). H-NS-dependent DNase I protection was also initiated near the E. coli gene silencer EAT6 (Fig. 9, lane 3), was spread in both directions, and eventually extended preferentially toward the promoter of the E. coli leuO gene (Fig. 9, lanes 1–3). Hence, both the S. typhimurium gene silencer AT8 and the E. coli gene silencer EAT6 are important for the promoter relay mechanism and are most likely the H-NS nucleation sites. This possibility was further tested using the replacement plasmid constructs where the S. typhimurium gene silencer AT8 was replaced with either
the *E. coli* gene silencer EAT6 (the case in pWU802ES) or a same size neutral DNA sequence (the case in pWU802NS). The previous functional studies (Figs. 6 and 7) clearly indicated that EAT6 was able to replace the gene silencing activity of AT8 in the *S. typhimurium* LCR-I for the repression of the promoter of the downstream leuO gene and even the more distal bla gene. Based on this observed functional replacement, we also anticipated observing similar H-NS nucleation on the replacement plasmid construct, pWU802ES. Indeed, the 39-bp EAT6 DNA sequence was the first region occupied by H-NS as evidenced in the DNase I footprinting experiment (Fig. 10, left panel). Although the DNase I protection was not as efficient as had been observed in the experiment using the original *S. typhimurium* LCR-I DNA sequence (Fig. 8), the H-NS-dependent DNase I protection also eventually extended toward the promoter of the leuO gene (Fig. 10, left panel, lane 1). The lower efficiency is most likely due to imperfect compatibility of the *E. coli* gene silencer EAT6 in the context of *S. typhimurium* LCR-I DNA. The less-than-perfect compatibility is evidenced on the DNase I-mediated cleavage pattern of the DNA of the replacement plasmid construct pWU802ES. There is a stretch of intensive DNase I-mediated cleavages located near the junction of the 5’ end EAT6 DNA sequence and the context of the *S. typhimurium* LCR-I (the dense cleavage pattern shown in Fig. 10, left panel). Nevertheless, the DNase I footprinting result demonstrated that the *E. coli* gene silencer EAT6 is able to replace the H-NS nucleation function of the *S. typhimurium* gene silencer AT8 in *S. typhimurium* LCR-I. The function that is replaced must be H-NS nucleation because the H-NS nucleation was not observed upon the replacement with a same size neutral DNA sequence in the context of *S. typhimurium* LCR-I in plasmid pWU802NS. Because the gene silencing activity observed in the functional assay (Fig. 6) correlates highly with the H-NS nucleation observed in the DNase I footprinting experiments (Figs. 8–10), we concluded that the gene silencer AT8 or EAT6, important for the promoter relay mechanism, must be an H-NS nucleation site rather than an ordinary H-NS-preferred binding DNA sequence.

**FIG. 9.** H-NS-dependent DNase I protection in *E. coli* LCR-I. A unique end radioactively labeled DNA segment consisting of the upstream region (~8 position to ~170 position) of *E. coli* leuO gene was generated by PCR. The end-labeled DNA was incubated with H-NS at a concentration indicated above each lane and exposed to DNase I (0.4 unit per reaction). The positions of relevant elements and restriction sites in the region are marked.

**FIG. 10.** H-NS-dependent DNase I protection required the presence of the DNA sequence of a bacterial gene silencer in *S. typhimurium* LCR-I. A unique end radioactively labeled DNA segment consisting of the promoter and the upstream region (~40 position to ~269 position) of the *S. typhimurium* leuO gene was generated by PCR using either pWU802ES or pWU802NS. The silencer AT8 in *S. typhimurium* LCR-I was replaced with the *E. coli* silencer in the PCR product when pWU802ES was used as the DNA template (left panel). The silencer AT8 in *S. typhimurium* LCR-I was replaced with a same size (47 bp) neutral DNA sequence in the PCR product when pWU802NS was used as the DNA template (right panel). The end-labeled DNA was incubated with H-NS at a concentration indicated above each lane and exposed to DNase I (0.5 unit per reaction). The positions of relevant elements and restriction sites in the region are marked.
promoter of leuO gene. Together, these data supported a nucleoprotein filament model for the H-NS effect.

The binding of a foreign protein, such as the lac repressor or λ repressor within the cis-spreading pathway of the transcriptionally repressive nucleoprotein filament, was capable of blocking the gene silencing effect found in the AT-rich DNA sequences flanking the promoter of the bgl operon (34). To confirm that a continuous cis-spreading nucleoprotein filament is responsible for the silencing activity found in our model system, a lac operator was positioned at the AatII site of the testing plasmid pWU802. Under this testing condition, we expected that the lac repressor should block the gene silencing activity from reaching the downstream bla promoter, whereas the leuO promoter remained as repressed (the model illustrated in Fig. 11) if a continuous cis-spreading nucleoprotein filament indeed simultaneously repressed the activities of both the leuO promoter and the bla promoter on the testing plasmid (as observed in Figs. 6 and 7). Indeed, we found that the presence of a lac operator at the AatII site derepressed the activity of the bla promoter but not the leuO promoter upon providing the trans-acting lac repressor (Fig. 11, lanes 2 and 8). If the inserted operator DNA was not positioned between the gene silencer and any one of the two target promoters (lac operator was positioned at the HindIII site of the testing plasmid as shown in Fig. 11, lanes 3 and 9), the lac repressor did not affect the gene silencing effect on either promoter. The binding of the lac repressor at the operator site rather than the inserted operator DNA must be responsible for the derepression of the bla promoter activity. This is concluded because no effect was observed if the lac repressor is not provided (Fig. 11, lanes 4–6). Therefore, it is clear that a cis-spreading nucleoprotein filament initiated from the gene silencer AT8 is responsible for the repression of leuO promoter activity. The transcriptionally repressive nucleoprotein filament further extended to the downstream region as a continuous nucleoprotein filament structure (model illustrated in Fig. 11) and resulted in the repression of the downstream β-lactamase gene on the testing plasmid.

FIG. 11. A cis-spreading nucleoprotein filament is responsible for gene silencer AT8-mediated transcriptional repression. The plasmid, pWU802, was tested in MC1060, a lac- strain. The lac repressor was expressed from the pACYC-based pSO1000 in MC1060 when necessary. The activities of the bla and leuO promoters on pWU802 were simultaneously monitored. The quantified promoter activity shown at the bottom of each lane for three repeated experiments is expressed as the mean within the range of ±0.02 (S.D.). Illustrated in the model is the effect of a tetrameric lac repressor located at either the AatII site or the HindIII site for blocking the cis-spreading nucleoprotein filament initiated from gene silencer AT8.

DISCUSSION

Clearly, the AT-rich LCRs in the ilvIH-leuO-leuABCD gene cluster are prone to form DNA secondary structures that are functionally important for transcription regulation (the promoter relay mechanism) in the region. Consequently, the DNA geometry rather than the specific DNA sequence of the LCRs was conserved between the two closely related enteric bacteria, E. coli and S. typhimurium. In the present study, the function of one of these conserved DNA geometrical elements was characterized. The characterization led to the finding that an H-NS-mediated cis-spreading nucleoprotein filament is responsible for the leuO gene silencing. H-NS is an abundant nucleoid protein important for the architecture of bacterial chromosomes (32, 35, 36). The protein is known to affect the expression of many genes including the hns gene itself (33, 37–39). Among them, the silencing mechanisms of proU and bgl genes have been relatively well characterized (34, 40–44).

It has been known for some time that H-NS binds to DNA in a sequence-nonspecific manner, but it preferentially binds with curved AT-rich DNA (45–47). Our finding is similar to the gene silencing activity mediated by the AT-rich DNAs found within the proximity of the proU and bgl promoters (34, 48). In these AT-rich DNAs, H-NS nucleation sites and flanking AT-rich DNAs are also important for their gene silencing activities (31, 40). In our model system, the flanking AT-rich DNA determines the directionality of the cis-spreading transcriptionally repressive H-NS filament structure toward the promoter of the target gene leuO. This is a perfect example that the exact function of a DNA element (the gene silencer in this case) shall be evaluated in its natural DNA environment (the neighboring AT-rich DNA in LCR-I in this case), as the gene silencer exerts a clear bi-directional transcriptional repressive activity in the testing plasmid pAO series where the gene silencer is surrounded by plasmid DNA context (13, 14). The previously demonstrated distance limit (300 bp) for the AT8-mediated gene silencing effect that was determined using pAO plasmids (13) may also be due to the flanking foreign DNA context. In the
present study with the assays performed in the presence of the surrounding LCR-I DNA sequence, AT8-mediated gene silencing was found to be able to extend to such a distance as to affect not only the target leuO promoter but also the promoter of the \( \beta \)-lactamase gene, \( \text{bla} \), which is located downstream on the testing plasmid of the pWU802 series (Figs. 6, 7, and 11). This could be related to a previous finding that the DNA sequence upstream of the \( \beta \)-lactamase gene also shows H-NS binding preference (48). This DNA sequence helps to extend the gene silencing effect to a further distance. Hence, the gene silencer AT8 (an H-NS nucleation site) serves as a crucial driving force for the regulation of the effect. This conclusion is basically similar with the finding in a previous study by using the repression of \( \text{proU} \) or \( \text{bgl} \) as a model system (31). It seems that nature has evolved the optimal transcription controls in the \( \text{ilvIH-leuO-leuABCD} \) gene clusters. The optimal transcription controls do not require DNA sequence specificity in the control region for either the \( \text{proU} \) promoter but also the promoter of the \( \beta \)-lactamase gene, \( \text{bla} \).

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