Repression of transcription initiation from the two gal promoters, P1 and P2, requires binding of GalR protein to two flanking operators, O2 and O1, binding of HU to a site, hbs, located between the two operators, and supercoiled DNA template. Previous experiments suggested that repression involves the interaction of two DNA-bound GalR proteins, which generates a 113-bp DNA loop encompassing the promoter region. Interaction between two DNA-bound proteins would be allowed if the binding sites on DNA are properly aligned. To test the idea that the observed repression of gal transcription in vitro is mediated by DNA looping, we investigated the effect of changing the relative angular orientation of O2 and O1 in the DNA helix. We found that repression is a periodic function of the distance between the two operator sites. Since repression recurred commensurate with DNA helical repeat, we conclude that the observed in vitro repression is mediated by DNA looping and the in vitro conditions reflect the in vivo situation.

DNA looping appears to be a general phenomenon in DNA transaction reactions e.g. replication, transcription regulation, site-specific recombination, DNA condensation, etc. In gene regulation, DNA looping is frequently involved in both transcription repression and activation. Looping occurs when two identical or different proteins bound to spatially separated specific sites on DNA interact directly or through a mediator or when a bidentate protein simultaneously binds to distal DNA sites. Depending on the strength of the above interactions, DNA looping may or may not require the aid of a DNA bending protein (frequently referred to as an architectural protein), which facilitates loop formation by binding to a locus between the two distal DNA sites. In the loop, the two binding sites on DNA may be oriented in parallel or in antiparallel orientation (1–3).

The feasibility and stability of such a DNA-multiprotein complex has been studied in several systems, including the gal, lac, and ara operons and bacteriophage λ of Escherichia coli. The complex formation depends on a variety of conditions: the proper angular orientation of the protein binding sites on DNA (4–8), the size of the loop (9), and the superhelicity of the DNA (10–12). Because shorter DNA residues torsional change (13), the proper angular orientation of the two binding sites is more important with relatively shorter loop size than with longer loop size. It has been suggested that a loop size of less than 150 bp strictly depends upon proper phasing of the protein binding sites, less so for 200 bp, and not at all for 400 bp and up. The precise size of the helix repeat also depends to a certain extent on the DNA sequence of the loop (9). Here we report the results of the effect of changing the relative angular orientation of the binding sites, which were separated by less than 150 bp in DNA, on transcription repression in the gal operon of E. coli (14). Previous results suggested that repression involves the formation of a DNA loop by the interaction of two GalR repressor molecules bound to specific operator sites (O2 and O1), which are separated by 113 bp (Fig. 1). The GalR-GalR interaction is facilitated by the binding of a histone-like protein HU to a DNA site (hbs) between the two operators (15–17). Our results showed that the two operators are located at an optimal angular orientation with respect to each other. Changing the distance even by one bp decreased repression efficiency. Repression efficiency changed with an increasing number of base pair additions or deletions in a periodic fashion commensurate with DNA helical repeat.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**All restriction endonucleases, alkaline phosphatase, and T4 polynucleotide kinase were purchased from New England Biolabs. Recombinant RNasin Ribonuclease Inhibitor (40 units/µl) was obtained from Promega. Denaturing polyacrylamide gel solutions (Gel-mix®8) and T4 DNA ligase were obtained from Invitrogen. DNA primers were purchased from Bioline Biotechnologies. DNA sequencing (dRhodamine) and XL PCR kits were supplied by Applied Biosystems. [α-32P]UTP (specific activity = 3000 Ci/mmol, 10 µCi/µl) and [γ-32P]ATP (specific activity = 7000 Ci/mmol, 167 µCi/µl) were obtained from ICN Pharmaceuticals.

**Plasmids—**The parental plasmid (pSA508) was constructed by Choy and Adhya (18). Briefly, it is a derivative of pBl24 (International Biotechnologies) and contains the following features: the λ attachment site (attP'), the corresponding bacterial attachment site (attB'), the Rho-independent transcription terminator site of rpoC, and multiple cloning sites (18, 19). Plasmid pSA5850 was generated by digesting pSA508 with EcoRI and PstI in the multiple cloning site region and inserting a PCR fragment, which contained the wild type gal regulatory region from −75 to +91 digested with the same enzymes. The recombiant plasmids were transformed into E. coli-competent cells (DH5α) obtained from Invitrogen. The plasmid DNA was purified from DH5α cells by following the protocol outlined in the Qiagen Midi Purification Kit. The DNA concentrations were determined spectrophotometrically at 260 and 280 nm. The relative ratio of supercoiled to relaxed DNA was 99:1 as determined by analyzing DNAs on a 1% agarose gel in 1× TAE buffer (40 mM Tris acetate and 0.1 mM EDTA (pH7.8)) and quantitating the bands on Eagle Eye™ II (Stratagene). The superhelical density of pSA580 preparation was −0.062 (data not shown).

When pSA5850 used in vitro as a template for transcription, P1 and P2 result in the synthesis of 125- and 130-nt long RNA, respectively. 1 PCR-generated DNA fragments with either base pair insertions or deletions were cloned into pSA5850 by replacement of the wild type gal 2 result in the synthesis of 125- and 130-nt long RNA, respectively. 1

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sequence as shown in Fig. 2. The relevant DNA sequence of the derivatized plasmids was determined by using ABI Prism 310 Genetic Analyzer.

Proteins—E. coli RNA polymerase was purchased from Amersham Biosciences. The fraction of σ-containing RNA polymerase was determined as described (20, 21). RNA polymerase (gift from Dr. Ding Jin), which was known to be 100% saturation with σ was used as a control to determine the σ content in the RNA polymerase preparations used in this study. RNA polymerase samples were analyzed on a 10% Tris-glycine SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The amount of σ to that of α in RNA polymerase was determined by scanning the gel and quantitating the bands on Eagle Eye™ II. The saturation level of σ was 84%. Since the RNA polymerase (20 nm) used in the in vitro transcription assays was in excess of the DNA (2 nm), the saturation level of α would not affect the results. GalR was purified as described previously (22). A binding constant of 0.4 nM was determined by a gel electrophoretic mobility shift assay on a 6% nondenatured polyacrylamide gel by titrating increasing amount of GalR on a DNA fragment of 238 bp containing a single operator Og located near the center (data not shown). The DNA fragment was labeled with T4 polynucleotide kinase and [γ-32P]ATP. The DNA binding activity was determined to be about 30%. HU purification followed the procedures of Aki et al. (23).

In Vitro Transcription Assays—In vitro transcription reactions were performed as follows: DNA templates (2 nm) were preincubated at 37 °C for 5 min in transcription buffer (20 mM Tris acetate, 10 mM magnesium acetate, 200 mM potassium glutamate) supplemented with 1 mM dithiothreitol, 1 mM ATP, 0.8 units of rRNasin and 20 nM RNA polymerase in a total reaction volume of 50 μl. When required, 80 nM GalR, and 80 nM HU were added. To start the transcription reactions, nucleotides were added to a final concentration of 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP, and 5 μCi [α-32P]UTP. The reactions were incubated for an additional 10 min before they were terminated by the addition of an equal volume (50 μl) of loading dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). Samples were heated to 90 °C for 2–3 min, chilled on ice, then loaded on an 8% sequencing gel and electrophoresed at constant power of 60 W in 1× TBE (89 mM Tris borate/2 mM EDTA (pH 8.3)). The gels were scanned on a PhosphoImager: 425: and the amount of transcripts were determined by using the “Next Valley” method of ImagequantNT. The RNAI transcripts (108 nt) were used as an internal control to quantify the relative amount of gal transcripts (24).

RESULTS

We have developed an in vitro system to study transcriptional regulation of the gal promoters by GalR and HU (18). In this system, simultaneous repression of the gal promoters by GalR is dependent upon the same conditions required in vitro (16): binding of GalR to both Og and O1, the presence of HU, and supercoiled DNA template (15). Such conditions have been proposed to generate a DNA loop, which is essential for repression. Binding of GalR to Og, in the absence of HU, compresses transcription initiation from the two gal promoters in the opposite direction; the regulator inhibits transcription initiation from P1, but enhances the same from P2 (18, 25). Og-bound GalR makes differential contacts with RNA polymerase at P1 and P2 to bring about the dual control (26). But in the presence of HU, GalR binding to both operators represses transcription from both promoters engendering, as proposed, a DNA loop (15, 23). Thus, the repression of P2 is totally dependent on the proposed DNA loop formation. We studied the spatial relationship of the two operators for the purpose of looping in our purified system by following the degree of P2 repression as an indicator of the efficiency of DNA looping in the gal system. To systematically study the relationship of the DNA spacing between the two operators and the efficiency of DNA looping, engineered DNA templates for in vitro transcription repression were generated, which either contained 1–21 additional base pairs or were deleted for 2–12 base pairs between Og and O1 at positions indicated in Fig. 2.

Base Pair Insertions—The DNA sequence of the insertions and their immediate vicinity are shown in Fig. 2A. Note that the inserted base pairs were between the promoters and O1 at position +32 relative to the transcription start site of P1 (1+); such alterations keep intact the dual regulatory relationship between Og and the promoters in the absence of DNA looping (27). The results of gal transcription on the DNA templates with insertions in the presence and absence of GalR and HU are shown in Fig. 3. The transcription from P1 and P2 promoters resulted in the synthesis of two RNA species differing in size by 5 nt in each template as expected (18). The base pair insertions between the promoters and O1, as expected, did not affect either the intrinsic transcription of P1 and P2 (Fig. 3, A–C, lane 1 and every third lane thereafter) or the opposite regulation of the promoters by GalR (Fig. 3, A–C, lane 2 and every third lane thereafter). Transcription from P1 was uniformly repressed in the presence of GalR alone in all of the templates because of the binding of the repressor to Og. However, an enhancement of transcription of the P2 promoter in the reactions was not apparent as expected; the amount of RNA synthesis from P2 seemed to be repressed in some of the cases, for example, in the DNA template that carried a 5-bp insertion (Fig. 3A, lane 17). The observed reduction in the amount of full-length P2 RNA in specific templates in the absence of HU was because of frequent transcriptional “road block” by GalR bound to Og, resulting in the synthesis of shorter P2 RNA molecules as indicated in Fig. 3.1 When both full-length RNA and RNA generated by the road block were taken into account, GalR bound to Og enhanced transcription from P2 in all of the templates tested except the one with 17 bp insertion (quantitative data not shown). The reason for the anomalous behavior of the template with 17 bp insertion is not known.

The results of transcription repression of P2 in the presence of HU on mutant DNA templates that contained additional base pairs between the two operators are shown in Fig. 3, A–C, lane 3 and every third lane thereafter. The amount of P2 transcription (both full-length and road-block) in the presence of HU relative to that in the absence of HU, reflecting the extent of looping in these templates, are shown graphically in Fig. 5. In the wild type template, P2 was repressed as expected. As Og was rotated around the DNA helix with respect to O1 by inserting 1–5 bp, P2 repression became progressively weaker with maximum derepression being observed in the case of the 5 bp insertion, indicating that the relative angular orientation of the two operators are critical for repression of P2. Moreover, the repression of P2 gradually restored when the distance between Og and O1 was further increased by 6–10 bp, restoration of repression being maximal with 10 bp addition. The periodic pattern of derepression and repression was repeated when the spatial separation between Og and O1 was further increased in the same stepwise manner to 21 bp. In other words, repression recurred in a periodic fashion with maxima when Og and O1 were separated by 113 bp or 123 bp, and with minima when the operators were 119 bp or 130 bp apart (see Fig. 5).
Fig. 2. The list of plasmid DNA templates, constructed in which base pairs were added (A) or deleted (B) incrementally by 1 to 2 bp. The additions were between position +32 and +33 as indicated by the arrow; the deletions started at position −50 and extended downstream to −39. The inserted base pairs are shown in thin letters, the deletions by dashes. The OE and OI sequences are boxed. The plasmid designation of each construct is shown on the left; pSA850, wild type is shown at the top.
FIG. 3. The results of gel electrophoresis of the gal RNA made in vitro from DNA templates with base pair insertions (details under “Experimental Procedures”) in the presence and absence of GalR and HU at the concentrations indicated. The numbers at the top show the number of base pairs inserted between OE and OI (see Fig. 2A). The arrows, marked P1 and P2, are RNA made from the two gal promoters; the arrow, marked RNA1, show the two RNA species made from a control promoter (rep) also present in the plasmids; the bracket, marked RB of P2, shows the RNA molecules made from P2 but prematurely terminated by GalR bound to OI. All templates used were supercoiled plasmids. (A) Wild type DNA templates and templates with 1, 2, 3, 4, and 5 bp insertions. (B) DNA templates with 6, 7, 8, 9, and 10 bp insertions. (C) DNA templates with 11, 13, 15, 17, 19, and 21 bp insertions.
Base Pairs Deletions—The DNA sequence of the templates with stepwise deletions are shown in Fig. 2B. It is important to point out that base pairs were deleted between \( O_E \) and the promoters, thus changing the response of transcription from \( P_1 \) and \( P_2 \) to GalR bound to \( O_E \) (27). In contrast to the results with base pair additions between the promoters and \( O_I \), the pattern of intrinsic transcription from \( P_1 \) and \( P_2 \) in the absence of GalR and HU in the templates with base pair deletions were somewhat different (Fig. 4, lane 1 and every third lane thereafter). Whereas transcription from \( P_2 \) was only slightly lowered or enhanced, transcription from \( P_1 \) was significantly lowered with most of the templates with base pairs deleted upstream for an unknown reason, although \( P_1 \) is located further downstream than \( P_2 \) relative to the deleted segments. We also note that because the angular orientations of the two promoters relative to \( O_E \) in these templates changed in a periodic fashion, the opposite regulatory effect of GalR bound to \( O_E \) upon transcription also changed in parallel (Fig. 4, lane 2 and every third lane thereafter) (27).

As expected, the transcription from \( P_2 \) was alternately enhanced and inhibited and that from \( P_1 \) inhibited and enhanced, by gradually changing the relative angular orientation between \( P_2 \) and \( O_E \). Because of these periodic variations, the potential for DNA looping was quantified by measuring the total amount of transcription from \( P_1 \) and \( P_2 \) in the presence of both GalR and HU (Fig. 4, lane 3 and every third thereafter) relative to that in the absence of GalR alone for each DNA template (Fig. 5). The results showed that, as in the case of base pair additions, progressive deletions of 2–12 bp between the two operators affected repression in a periodic fashion; for example, repression was lifted by deletion of 5 bp and reoccurred by removal of 10 bp (Fig. 5).

DISCUSSION

A structure-based genetic study of the interaction of the two operator-bound GalR dimers defined the GalR dimer interface (3). The interfacial interaction results in a stacked V-shaped, tetramer. Stereochemical model building of the proposed loop in \( gal \) DNA and the evaluation of the DNA elastic energies strongly supported a DNA loop in which the two operator-bound GalR dimers, stacked in the V-shape, adopts an antiparallel orientation. The results presented here show a periodic dependence of repression of \( gal \) operon transcription with incremental (both increasing and decreasing) changes in the length of the DNA between the two GalR binding sites. The observed periodicity (Fig. 5) is identical to the helical repeat of B-DNA, showing that our \textit{in vitro} regulatory system engenders transcription repression, as proposed, by DNA looping in the presence of GalR and HU. The role of HU in assembling the loop in \( gal \) is architectural and involves a protein-protein contact between HU and GalR (3, 28). In addition to HU, DNA looping by GalR also needs supercoiled DNA template both \textit{in vivo} and \textit{in vitro} (16, 23). It is conceivable that such requirements provide architectural help because the two operators in the wild type DNA are not spaced apart favorable for efficient looping. However, none of the DNA templates, engineered to have incrementally increased or decreased distance between the two operators, became independent of HU (as described here) or the requirement of supercoiled DNA (data not shown). Thus, the distance of 113 bp between \( O_E \) and \( O_I \) in wild type is not a suboptimal situation requiring architectural help from HU and/or DNA supercoiling.

DNA looping as a periodic function of the DNA length between the two GalR binding sites also allowed estimation of the size of a DNA helical turn under \textit{in vitro} conditions to be $-10$
FIG. 4. The results of gel electrophoresis of the gal RNA made in vitro from DNA templates with base pair deletions. The details are as in the legend to Fig. 3. The negative signs reflect the base pairs deleted between $O_E$ and $O_I$ (see Fig. 2B).

FIG. 5. Dependence of repression of in vitro transcription on the distance in base pairs between $O_E$ and $O_I$. The wild type distance (arrow) is 113 bp. The results of P1 and P2 transcription taken together are shown for base pair insertions (Fig. 3) (circles) and base pair deletions (Fig. 4) (squares) for reasons discussed in the text. The amount of gal transcription was normalized to the control RNA1 and to the number of uridine residues in each gal transcript, since $[\alpha-^{32}P]UTP$ was used as a label for the transcription assays. The relative amount of gal transcription reflecting the efficiency of DNA looping was determined by dividing the amount of RNA in the presence of GalR by that in the presence of GalR and HU.
bp. A helix repeat of 10.5 bp has also been found in DNA looping by bacteriophage 
Al and E. coli LacI repressors in vitro (6, 7). Dependence of loop formation on the relative angular
orientation of the two protein binding sites in a periodic fashion
and thereby estimation of the size of the helix repeat has also
been studied in several systems in vivo including an artificial
construct in which a heterologous promoter was spanned by gal
operators, O_6 and O_1 (4, 8, 10, 29, 30). The size of a DNA helical
turn by in vivo estimation was shown to be within 11.0–11.3
bp. The in vitro results of DNA helix size, based upon protein-
mediated DNA looping, are consistent with the estimation of
the helix size of about 10.5 bp by different in vivo assay systems in other cases using both circular and linear DNA
(31–34). Thus, there is a discrepancy of about one base pair in
helix size of DNA structure in vitro and in vivo. The reason for
the difference needs further investigation.

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In Vitro Repression of the gal Promoters by GalR and HU Depends on the Proper Helical Phasing of the Two Operators

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