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Control of gal transcription through DNA looping: Inhibition of the initial transcribing complex

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ABSTRACT Involvement of DNA looping between two spatially separated gal operators, O9 and O1, in repression of the gal operon has been demonstrated in vivo. An in vitro transcription assay using a minicircle DNA containing the gal promoter region with lac operators was employed to elucidate the molecular mechanism of repression. Wild-type lac repressors (LacI* protein molecules), which are capable of associating into a tetramer and forming a DNA loop, repressed transcription from promoter sites P1 and P2, whereas a non-looping lac repressor mutant (LacIad) failed to show normal repression of both of the gal promoters. Thus a DNA loop is also required for repression of transcription in vitro. Repression mediated by DNA looping resulted in the inhibition of the synthesis of complete as well as aborted transcripts, demonstrating that the repressive action was on the formation or activity of the initial transcribing complex. Under similar conditions, the gal repressor (GalR protein) did not repress the gal promoters effectively, apparently because it failed to loop DNA containing gal operators in the purified system. The component(s) or conditions that aid GalR in DNA looping remain to be identified.

Since topologically superhelical conformations of DNA have been shown to enhance the interactions between LacR and other transcriptional regulatory proteins to their cognate DNA sites in the formation of DNA loops (10-12), the effect of repressors on gal transcription was studied by using supercoiled “minicircle” DNA templates (unpublished work). These DNA minicircles, containing only gal and no other promoters, greatly facilitated investigating the mechanism of repressor action on the synthesis of both aborted and full-length transcripts from the gal promoters in the same assay. We report that (i) repression of the gal operon in vitro requires an interaction between repressors bound to two operators; (ii) repression occurs at a step prior to formation of the first phosphodiester bond; and (iii) while LacI represses both P1 and P2, repression by GalR, as opposed to the in vivo result, is incomplete for P1 and totally ineffective for P2.

MATERIALS AND METHODS

Plasmid and Bacterial Strains. Construction and functional elements of the plasmids used to generate supercoiled “minicircles” in vivo will be reported elsewhere. Briefly, the parental plasmid carried a multiple cloning site into which the gal promoter segment was inserted. This was followed by a transcription terminator. The promoter–terminator region was located on the plasmid between the λ phage attachment site, attP, and the corresponding bacterial site, attB. DNA minicircles carrying the gal promoter followed by the transcription termination were generated by site-specific recombination between the attP and attB sites in vivo in a host (SA1751) that provided the λ integrase and the host integrase factor, IHF. The minicircles were extracted and purified by gel electrophoresis (unpublished work).

pSA508, the parental plasmid, contained no promoter at the multiple cloning site. pSA509 contained a 288-base-pair (bp) segment of gal promoter (~197 to +91) cloned between the EcoRI and Pst I sites of pSA508 (2). pSA510 was identical to pSA509 except that both gal operators, O9 and O1, were replaced with the consensus lac operator sequence, 5'-TGTGAGCGCTCAAA-3' (5). pSA511 and pSA512 were also identical to pSA509 except that the internal operator (O9) or the external operator (O1) were replaced with the lac operator sequence, respectively. In the experiments of Fig. 5, an O8 allele served as an O7 allele for GalR. E. coli K-12 strain SA1751 is F- strR lac- trp- Δ(chlD–pgl)320 [λ zis-c857 Δ(cro . . .) chlE]MI.

Purified Proteins. Wild-type gal repressor (GalR*) and wild-type (LacI*) and mutant (LacIad) lac repressors were purified as described (6, 13). CRP was purified by FPLC (Pharmacia) from an E. coli strain carrying the crp+ gene on a multicopy plasmid, pHA5 (gift of S. Ryu and S. Garges).

Abbreviations: CRP, cAMP receptor protein; IPTG, isopropyl B-D-thiogalactopyranoside.

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RNA polymerase (100% saturated with σ factor) was from Epicentre Techniques, Madison, WI.

Transcription Assays. Transcription reactions were carried out by a procedure to be described in detail elsewhere. Briefly, 2 nM DNA template, 0.1 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP, and 10–20 μCi of [α-32P]UTP (1 Ci = 37 GBq) were preincubated in buffer (20 mM Tris acetate, pH 7.8/10 mM magnesium acetate/100 mM potassium glutamate) at 37°C for 5 min. When the variable components such as CRP, repressor, cAMP, or inducer were present, they were included in the preincubation mix at concentrations described in the figure legends. Transcription was initiated by the addition of RNA polymerase (20 nM) in a total volume of 50 μl and was terminated after 10 min at 37°C by the addition of an equal volume (50 μl) of RNA loading buffer [80% (vol/vol) deionized formamide/1× TBE (89 mM Tris/89 mM boric acid/2 mM EDTA)/0.025% bromophenol blue/0.025% xylene cyanole]. The mixture was heated at 90°C for 2 min and electrophoresed in 8 M urea/8% polyacrylamide sequencing gels (40 cm × 0.4 mm). The RNA transcripts were quantitated by determining cpm with an Ambis β scanner.

RESULTS

Transcription from gal Promoters in DNA Minicircles. In vitro gal transcription was studied using supercoiled DNA minicircles that contained only the overlapping gal promoters along with its cognate regulatory elements (Fig. 1). The gal promoter segment in the plasmid DNA was followed by a factor-independent transcription termination signal that terminated the synthesis of gal transcripts at a specific site. Since the minicircles contained no other promoter, it was also feasible to study directly the effect of repressor on abortive initiation products made during gal transcription (unpublished work). Various DNA minicircles were made in vivo as described in Materials and Methods. gal promoter-directed transcription by purified RNA polymerase on DNA minicircle templates containing the gal promoters was monitored in the absence or presence of 0.2 mM CAMP and excess (100 nM) CRP. The transcription products were electrophoresed in denaturing 8% and 25% polyacrylamide gels to analyze full-length and abortive transcripts, respectively.

![Figure 1](image1.png)

**Fig. 1.** gal promoter of *E. coli* in the minicircle generated from pSA509. Oe and Oi represent external and internal operator, respectively; ter is the transcription terminator.

Supercoiled DNA minicircles (pSA509) containing the wild-type gal promoter segment yielded two distinct full-length transcripts in the absence of cAMP: a 120-nucleotide-long RNA from the P1 promoter; and a 125-nucleotide-long RNA from P2, as seen in an 8% gel (Fig. 2A, lane 3). In the presence of 0.2 mM CAMP, the P2 RNA band disappeared, and the P1 RNA band was enhanced 3-fold (lane 4). DNA minicircles without a promoter (pSA508) did not show any transcript in the absence or presence of cAMP (Fig. 2A, lanes 1 and 2). Electrophoresis of products from the same transcription reaction in a 25% polyacrylamide gel showed the characteristic aborted transcripts from the gal promoters (Fig. 2B, lanes 3 and 4; ref. 14; unpublished work). In the absence of CAMP, the predominant aborted transcripts, trimers and hexamers, were made primarily from the P2 promoter. In the presence of CAMP, aborted transcripts were

![Figure 2](image2.png)

**Fig. 2.** *In vitro* transcription in the presence or absence of 0.2 mM CAMP with the vector minicircle (pSA508) or *gal* minicircle (pSA509) as DNA templates. CRP was present at 100 nM. Transcription products were analyzed in denaturing 8% (A) and 25% (B) polyacrylamide gels.
exclusively expressed for P1, although in very small amounts. As expected, the minicircles without a promoter did not produce any aborted transcripts (Fig. 2B, lanes 1 and 2).

Repression of gal Transcription by Wild-Type and Mutant lac Repressors. Repression of gal transcription was studied using minicircle gal DNA template (pSA510), containing lac operator sequences in place of the gal operators at $O_1$ and $O_2$ ($O_2^t$ and $O_1^t$, respectively) (5). Two types of lac repressor were used to study repression: wild-type LacI* and mutant LacI$d^d$ repressors. LacI* forms a tetramer and represses gal operon containing the lac operators in vivo, whereas the mutant repressor is a non-tetrameric protein and fails to repress under similar conditions (6). The mutant repressor exhibits normal binding to operators but fails to carry out the association of the dimer units into a tetramer as judged by electron microscopy (6, 15) and electrophoresis of operator-repressor complexes (16). Fig. 3 shows the effect of LacI* on gal transcription in vitro in the absence (lanes 1–8) and presence (lanes 9–16) of 0.2 mM cAMP. Increasing concentrations of LacI* protein repressed both P1 in the absence or presence of cAMP and P2 in the absence of cAMP (Fig. 3, lanes 1–4 and 9–12). Quantitation by direct scanning of the radioactivity of the full-length transcripts in the gels is shown graphically in Fig. 3C. LacI* was able to repress gal transcription by >95%. We also observed similar repression by LacI* of the aborted transcripts made from the gal promoters (Fig. 3B). In the absence of cAMP, LacI$d^d$, however, repressed P1 by only about 75% and P2 by <10% (Fig. 4A and B; lanes 1–4). In the presence of cAMP, P1 was repressed by LacI$d^d$, again by 75% (lanes 9–12). The results are shown quantitatively in Fig. 4C. Interestingly, the inhibition of P2 activity by cAMP-CRP was relieved slightly by LacI$d^d$. This is more apparent for the aborted transcripts (trimer and hexamer) from P2 (Fig. 4B, lanes 1–4 and 9–12). The repression of P1 and P2 by LacI* and the weak repression of P1 by LacI$d^d$ were totally relieved, as expected, by the addition of 1 mM IPTG, which inactivates lac repressors, showing that the inhibition of transcription was lac repressor-specific (lanes 5–8 and 13–16 in Figs. 3 and 4). Note that the repression by LacI* was almost complete in every case, with half-maximal repression obtained at ≈10 nM repressor (Fig. 3C). The maximal repression of P1 transcript by LacI$d^d$ of only 75% required very high repressor concentration (90 mM), with a half-maximal concentration of about 60 nM (Fig. 4C). Further increase of LacI$d^d$ repressor concentration had a nonspecific inhibitory effect: the inducer (IPTG) failed to derepress.

Property of gal Repressor. The effect of GalR* on transcription was investigated in vitro in the absence or presence of 0.2 mM cAMP and 100 nM CRP by using the minicircle DNA template containing wild-type gal operators (pSA509) at $O_2$ and $O_1$ ($O_2^t$ and $O_1^t$, respectively). The synthesis of both full-length and aborted gal RNA was investigated under these conditions. Quantification of the full-length transcripts is
shown in Fig. 5A. In the presence of cAMP, where only P1 was transcribed, ~80 nM GaIR+ was able to reduce the P1 transcripts only about 80% (Fig. 5A Right). P2 transcripts, both abortive and full-length, were undetectable in the presence of cAMP but increased slightly with increasing GaIR+ (results for aborted transcripts not shown). In the absence of cAMP, the RNA band from P1 was reduced by GaIR+ about 60% (Fig. 5A Left). Thus, a significant amount of gal transcription from P1 was maintained in the presence of saturating concentrations of GaIR+ irrespective of the presence of cAMP. We found that P2 activity was not repressed by GaIR+. In fact, P2 activity increased slightly. The poor repressive effects of GaIR+ on P1 in the absence and presence of cAMP were abolished by the addition of 10 mM D-galactose (results not shown).

**Mutation Operators.** The amount of partial repression by GaIR+ described above is roughly equivalent to that obtained in vivo with an intact O6 (O6) and mutant O1 (O1') (5). To investigate whether the partial repression in the purified system was due to an interaction between GaIR+ and O6 (O6') alone, DNA minicircle templates with mutations at either O1 or O6 were used to study the nature of repression with GaIR+ in vitro. Fig. 5B shows the effect of GaIR+ concentrations on the repression of transcription using minicircle DNA (pSA511) with the genotype O6EF. This mutant DNA (pSA511) and the wild-type DNA (pSA509) provided results that were more or less indistinguishable from each other: P1 transcripts were repressed about 80% at about 80 nM GaIR+ both in the presence and in the absence of cAMP, and P2 in the absence of cAMP was not repressed. The gal O6EF DNA (pSA512) showed no discernable effect on P1 and P2 (Fig. 5C). Even in the presence of very high GaIR+ concentrations, very little change of gal RNA synthesis was observed in either the absence or the presence of cAMP.

**DISCUSSION**

To elucidate the mechanism underlying the repression of transcription initiation from gal promoters, we have used supercoiled DNA minicircles that contain only the gal promoter and adjoining DNA control elements. Since these unitary promoter templates do not carry any other transcription initiation sites, all aborted and full-length transcripts originate from the gal promoter, allowing their direct qualitative and quantitative analysis by gel electrophoresis.

In agreement with the results obtained in vivo (17, 18), the complete transcripts in this system from P1 and P2 were synthesized at about equal efficiencies in the absence of cAMP-CRP. The presence of cAMP-CRP inversely regulated the activities of P1 and P2. cAMP activated P1 and inhibited P2 transcription, thus making the system totally physiological and suitable for further regulatory studies. In addition to the synthesis of the complete transcripts, much transcription aborted after making tri- to hexamers (14). A large majority of the aborted oligomers initiated mainly at P2. Thus this system allowed the study of the level at which repressor brings about inhibition of RNA synthesis in the gal system.

A requirement of DNA looping mediated by repressor bound to the spatially separated operator elements, O6, and O1, for repression of both P1 and P2 has been demonstrated in vivo (5, 6). Furthermore, GaIR has been suggested to inhibit transcription from the gal promoters at a step following RNA polymerase binding (7, 8). The use of the purified system has made two points. First, we have shown that LacI+, which shows DNA looping by electron microscopy (6, 19) and gel electrophoresis (16), results in normal repression of P1 and P2 on a gal DNA template containing lac operators. LacI+, a non-looping repressor mutant, shows incomplete repression of P1 and no repression of P2. Our results argue convincingly that the complete repression requires an interaction of the repressors bound to the operators rather than the mere occupation of the operators by the repressors.

Second, we showed that LacI+ established complete repression of not only full-length but also abortive transcripts (Fig. 3B), which implies that the repressor inhibits the first phosphodiester bond formation or a step prior to that—i.e., the formation or the activity of the initial transcribing complex (20). Incidentally, study of the mechanism of repression of the lac promoter by LacI+ in purified systems has led to
conflicting conclusions. Although it was originally believed that LacI* acts by inhibition of closed complex formation in the lac operon (21), it has been suggested that with wild-type lac promoter the repressor blocks a step after the formation of a closed promoter complex (22). More specifically, from studying the cAMP-independent lacUV5 promoter at high concentrations of LacI* and RNA polymerase, Straney and Crothers (23) suggested that LacI* blocks the isomerization of the RNA polymerase–lac promoter complex to an open form. In contrast, Lee and Goldfarb (24) have concluded that in the lacUV5 promoter, LacI* creates a kinetic barrier in the promoter clearance step, which leads to an idling and abortive transcript-producing RNA polymerase.

It is plausible mechanistically that a DNA loop structure “locks” the RNA polymerase at the promoter, thereby establishing repression without any direct contact between repressor and RNA polymerase. Alternatively, repression can be established through communication(s) between repressor and allosteric site(s) in RNA polymerase as part of a nucleoprotein complex (6, 8). Such contacts in the complex make RNA polymerase assume, in repression, an idle form. DNA looping juxtaposes the necessary components in a geometrically proper complex.

Interestingly, the repression of gal DNA expression by GalR+ was incomplete in the purified transcription assay. The P1 promoter activity was repressed 80% or less, while P2 promoter activity was not inhibited at all. This behavior of GalR+ resembles remarkably the pattern of repression brought about by the non-tetrameric, looping-defective LacI+ mutant (6) and suggests that purified GalR+ protein suffers the same non-looping property that LacI+ mutant protein possesses. Both LacI+ and GalR+ bind to operators normally as dimers but fail to associate into a tetramer–operator complex (13, 16, 25). Under the conditions used in our transcription assays, the corresponding operators are fully occupied by LacI+ and GalR+ (16, 24). Since GalR+ has been shown to repress effectively both P1 and P2 in a crude cell extract (S-30) system (26), we conclude that an element or a feature of the cell extract is missing in the purified system. The missing component or condition very likely aids association of dimeric repressor bound to operator to form DNA looping in the purified system. In this regard, GalR+ is different from LacI+, which can be tetramerized by itself through a leucine minizipper (27). LacI+ used in this study, is missing the C-terminal end containing the leucines. GalR+ does not appear to have the corresponding leucines. Since the dimeric LacI+, unlike GalR+, fails to repress in vivo, we presume the proposed looping-aid for GalR+ does not help LacI+.

A partial repression of P1 but not of P2, which was observed for GalR+ or LacI+, was also observed with GalR+ on the gal DNA carrying an intact O2 and a mutant O1. Since no repression was observed for a mutant O2 and intact O1 template, these results clearly show that the partial repression of P1 by GalR+ with intact operators was not the consequence of repressor interaction with both O2 and O1. Rather, the partial repression resulted from the occupation of O2 alone by a dimeric repressor. This is in agreement with the observations made in vivo and in the crude system that an intact O2 alone allowed a partial repression whereas intact O1 alone allowed fully constitutive expression of the operon even in the presence of saturating concentrations of repressor (5, 26). Alternatively, the partial repression does not involve the DNA loop. In an abortive transcription initiation assay, gal repressor has been shown to cause a 10% decrease in the open complex formation at P1, with a concomitant and similar amount of increase at P2 both in the absence and in the presence of cAMP-CRP (28). Consistent with our results, the minor repression of P1 and activation of P2 by GalR+ observed by Goodrich and McClure (28) need only an intact O2. We do not understand the significance of P2 stimulation by repressor. However, the weak repression of P1 achieved by GalR+ or LacI+ as we have observed in the absence of DNA looping, may not be through a completely different mechanism. In the framework of the repressor–RNA polymerase contact model described above, it is likely that RNA polymerase bound to P1 contacts the repressor bound to O2, but the repressive contact signal, in the absence of DNA looping, is not strong enough to establish complete repression.

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