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Specific Binding of *lac* Repressor to Linear versus Circular Polyoperator Molecules†

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ABSTRACT: Gel shift assays were used to examine the binding of the lactose (*lac*) repressor to polyoperator DNA molecules. Specific binding was differentiated from nonspecific DNA association by (i) equilibrating repressor–operator complexes below the nonspecific association constant and (ii) demonstrating the effects of the inducer isopropyl β-D-thiogalactoside (IPTG) on the formation of repressor–operator complexes. With the linear polyoperator molecules, all eight operator sites could be simultaneously bound by distinct repressors. However, with circular molecules, the eight operator sites were saturable by repressor only in the nicked circular state and not in the covalently closed circular form. Under the experimental conditions used, there was no evidence of bifunctional repressor binding or loop formation. The results suggest that the conformational perturbation of DNA that occurs upon specific repressor binding was retained in topologically closed molecules and could modify other operator sites so as to make them unavailable for specific binding.

Because many regulatory proteins have been reported to induce structural distortions in their target DNA sequences, such as bending, untwisting, or looping (Kotlarz et al., 1986; Dunn et al., 1984; Hatfull et al., 1987; Koudelka et al., 1988), one determinant of protein–DNA interactions may be local conformation. The *lac* repressor–operator system has long been a paradigm for investigation of sequence-specific interactions that regulate initiation of transcription. *lac* repressor is known to bind tighter to supercoiled DNA than to relaxed circular DNA (Wang et al., 1974; Sadler et al., 1977; Whitson et al., 1987). Additionally, specific binding is characterized by a conformational change of the operator DNA, a result of either helix unwinding, DNA bending, or a combination of the two (Wang et al., 1974; Kim & Kim, 1983). Assuming one repressor bound per operator site, an unwinding of approximately 55° per specific repressor–operator interaction can be calculated from the equilibrium binding of repressor to plasmids containing 15 operator sites (Kim & Kim, 1983). However, kinetic analysis of similar polyoperator plasmids suggests that only a single repressor can be accommodated on adjacent multiple binding sites until the number of operators comprising that target exceeds four (Sadler et al., 1980). This study utilized nicked circular plasmid DNA in which no apparent steric or structural features blocked the additional operator sites.

A variety of complex geometries have been inferred for the binding of *lac* repressor to its operator, depending on the concentration of protein and the size, length, and configuration of the operator fragment. A single *lac* repressor can bifunctionally bind two appropriately separated operators in a linear DNA fragment (Kramer et al., 1987; Whitson et al., 1987). The resultant looped protein–DNA complexes show retarded

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migration in gels and greatly decreased dissociation rates when assayed by the filter-binding assay. For circular fragments containing two operators, low positive or negative supercoiling changes the spacing requirements for repressor-mediated DNA loop formation (Kramer et al., 1988).

This paper describes a qualitative examination of the binding of multiple repressors to polyoperator molecules, utilizing a gel shift assay to visualize specific repressor–operator complexes (Fried & Crothers, 1981; Garner & Revzin, 1981). To minimize the effects of nonspecific affinity of repressor for DNA, the linear and circular molecules contained primarily operator sequences: 26 bp of each 36-bp operator were strictly homologous to the natural *lac* operator sequence.

Experimental Procedures

Strains, Enzymes, and Materials. *Escherichia coli* K12 strain HB101 (*lacI*$^+$*O*$^+$*Z*$^+$*Y*$^-$ *recA*) was used for cloning and amplification of plasmid DNA. Methods for preparation and analysis of plasmid DNA, transformation of *E. coli*, and repressor purification have been described (Sadler et al., 1980). DNA molecules were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described (Sadler et al., 1983). The concentration of active repressor was determined by titrating labeled 40-bp operator DNA at $<5 \times 10^{-10}$ M according to the nitrocellulose filter binding assay (Sadler et al., 1983).

Polyoperator Fragment Circularization. Circular DNA molecules were produced by ligation of DNA fragments (Shore et al., 1981). The 292-bp polyoperator fragment was ligated into monomeric circles at concentrations of <50 ng/mL (<4 $\times 10^{-10}$ M ends), a high concentration of T4 DNA ligase being used (ca. 0.4 unit/μL; New England Biolabs). Circularization reactions were incubated at 25 °C for 6–12 h; the reaction buffer was 50 mM Tris-HCl (pH 7.8) 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, and 50 μg/mL bovine serum albumin. Nicked circular molecules were produced by two methods. In the first, excess unlabeled ATP was omitted from the 5′-end phosphorylation reaction: circularization of the radiolabeled fragments resulted in a mixed population of covalently closed and nicked circular molecules. The second method utilized *Eco*RI nicking of covalently closed polyoperator circles in the presence of 100 mM ethidium bromide (Österlund et al., 1982). Linear, nicked circular, and covalently closed circular molecules were separated by electrophoresis in 2% (w/v) agarose–7 μg/mL chloroquine gels (Gellert et al., 1983).

Repressor Binding and Electrophoresis. Gel electrophoresis was used to resolve the complexes arising from multiple repressors binding to DNA fragments. The initial studies of the 207-bp *lac* promoter-operator (*lacP-O*) fragment and a 201-bp nonoperator fragment utilized a Tris–borate–polyacrylamide gel system (10 mM Tris, 16 mM borate, 1 mM Na_2EDTA) (Fried & Crothers, 1981) modified to pH 7.6. The multiple repressor complexes with the 292-bp polyoperator molecule were resolved in 1.4% (w/v) agarose gels containing Tris–acetate buffer (15 mM Tris, 20 mM sodium acetate, 2 mM Na_2EDTA; pH 7.6). Protein titration of the labeled DNA fragments (at DNA concentrations of 10^{-8} or 10^{-10} M) involved the addition of increasing amounts of repressor in a total final volume of 10 μL. The reactions were equilibrated for 30 min at 25 °C in the Tris–borate or Tris–acetate gel running buffer. Just prior to electrophoresis, 3 μL of glycerol/dye buffer [25% (v/v) glycerol and 0.05% (w/v) bromophenol blue in gel running buffer] was added to the sample, which was mixed and immediately loaded onto the gel under a field strength of 6–7 V/cm. After all samples were loaded, the voltage was reduced to 2–3 V/cm, and the protein–DNA complexes were electrophoresed for 6–8 h at 25 °C. The gel was dried onto Whatman 3MM paper, and the bands were visualized by autoradiography.

Table I: Operator and Polyoperator Plasmids

plasmid	vector	*Eco*RI fragment (bp)	repressor binding sites per fragment	internal *Hae*III sites in polyoperator	operator size (bp)
pOE203	pBR322	207	1[a]	0	207
pOE138	pMB9	40[b]	1	0	2 × 40
pOE152	pMB9	76	2	1	2 × 38
pHE4	pMB9	148	4	3	2 × 38, 2 × 36
pHE148	pBR322	148	4	3	2 × 38, 2 × 36
pHE8	pMB9	292	8	7	2 × 38, 6 × 36
pHE292	pBR322	292	8	7	2 × 38, 6 × 36
pHE580	pBR322	580	16	15	2 × 38, 14 × 36
pHE1156	pBR322	1156	32	31	2 × 38, 30 × 36

[a] This fragment contains sequences −140 to +63 of the *lac* operon, including the *lac* operator (−7 to +28) and *lacI-P* pseudooperator (−92 to −72) (Gilbert et al., 1976). [b] This operator is homologous to bp −5 to +22 of the natural operator (Figure 1; Sadler et al., 1980).

Results

Construction of Polyoperator Molecules. Plasmid pOE183 carrying two 40-bp operator sequences (Sadler et al., 1980) was subjected to partial *Eco*RI digestion followed by S1 nuclease digestion and ligation (Figure 1). The resultant plasmid carrying the dimer operator with the central *Eco*RI site deleted, pOE152, was identified by restriction analysis. The internal *Hae*III site was verified by digestion of the purified *Eco*RI fragment (Figure 1). The 76-bp dimer operator fragment was self-ligated, and a plasmid carrying a stable direct repeat tetramer operator was isolated. Repetitions of the cleavage and ligation cycle resulted in plasmids containing *Eco*RI-ended polyoperator fragments with 4, 8, 16, or 32 operators in direct repeat orientation (Table I).

Circularization of Polyoperator Fragments. Since the probability of ring closure for smaller DNA fragments depends on DNA chain length (Shore et al., 1981; Horowitz & Wang, 1984), the 292-bp eight-operator fragment seemed a logical choice for circularization. Attempts to ligate the 292-bp fragment into monomeric circles at DNA concentrations of 200 ng/mL were unproductive: the predominant ligation products were higher molecular weight multimers. Larger polyoperator molecules were constructed (Table I), which circularized more readily; however, the resultant multiple repressor–operator complexes proved more difficult to resolve. The fact that the 292-bp fragment did not readily circularize at DNA concentrations reported by others suggested that these fragments might have some unusual character which retarded covalent closure, e.g., reduced flexibility of the polymer due to its specific repeating sequence. Monomeric circularization of the 292-bp fragment was finally achieved by ligation at DNA concentrations of <25–50 ng/mL (Figure 2A). Populations of exclusively covalently closed circles or exclusively nicked circles were prepared by two methods (Figure 2B), as detailed under Experimental Procedures. Because neither the linear nor the circular polyoperator molecules exhibited unusual electrophoretic patterns in polyacrylamide or agarose gels (Gellert et al., 1983), their repeating sequences apparently did not cause any marked perturbation of the normal helical DNA structure.

Repressor Binding to Single Operator Molecules. Figure 3A shows complexes formed by *lac* repressor with a 207-bp *lacP-O* fragment that contains the natural operator sequence and the weak *lacI-P* pseudooperator sequence (Gilbert et al.,

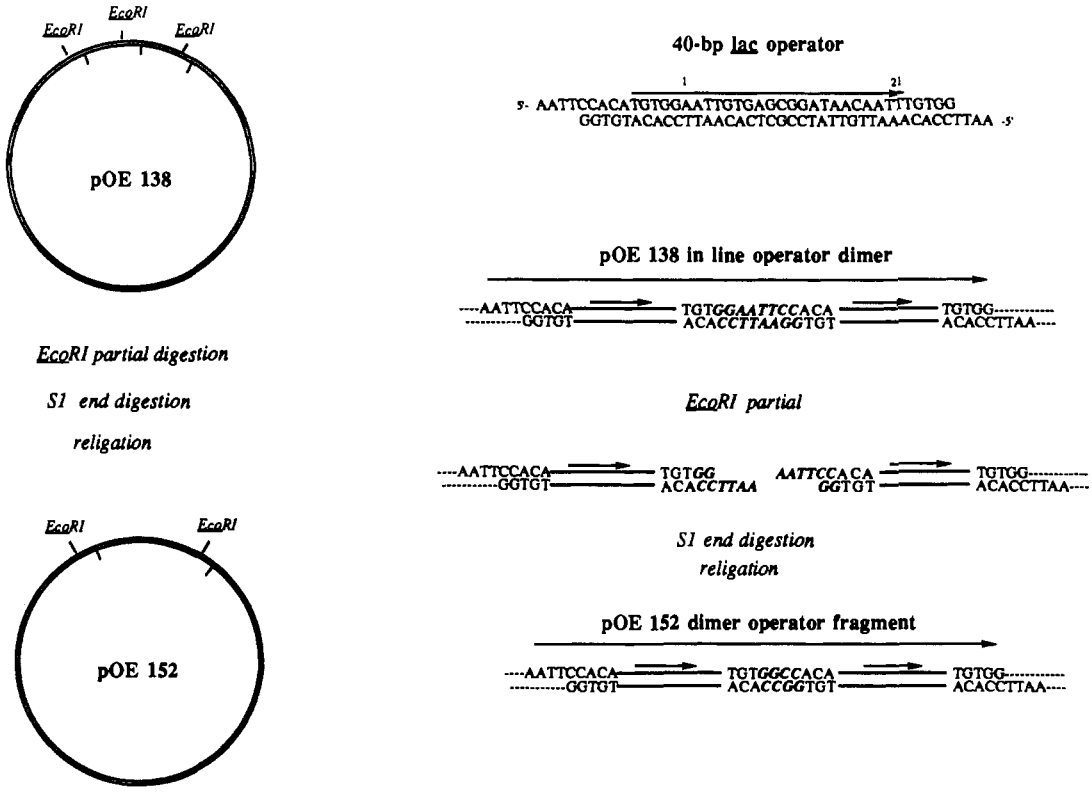

FIGURE 1: Construction of polyoperator fragments. (Left) Plasmid pOE138 carries a direct repeat dimer of a 40-bp *lac* operator (Sadler et al., 1980). Partial *Eco*RI digestion of pOE138, followed by nuclease S1 digestion and religation, constructed plasmid pOE152, which carries an *Eco*RI-ended dimer operator fragment with *Hae*III sites between the operators. (Right) The sequence of the *Eco*RI-ended 40-bp operator is given above the details for conversion of the *Eco*RI sites between tandem operators to *Hae*III sites. The arrow over the 40-bp sequence indicates homology to the natural operator, and position 1 indicates the start of transcription of *lacZYA* mRNA.

1976). At a DNA concentration of 10^{-8} M (Figure 3A), repressor not only specifically bound the operator (DR_1) and the pseudooperator (DR_2) but also nonspecifically associated with the remaining DNA (complexes DR_3–DR_8), as previously reported (Fried & Crothers, 1981). At a DNA concentration of 10^{-10} M (Figure 3B), repressor bound primarily the operator site (DR_1), although with increased concentrations (repressor: fragment ratios of >6) it also bound the pseudooperator site (DR_2). The absence of the nonspecific loading seen at 10^{-8} M DNA indicated that, at concentrations below its nonspecific affinity constant of ca. 10^{-8}–10^{-9} M, repressor did not associate sufficiently with the nonoperator DNA sequences to form unique complexes in this gel system.

Specific Binding versus Nonspecific Association. To assess nonspecific loading of the repressor on nonoperator DNA in these gel systems, a 201-bp *Hpa*II fragment of pBR322 (nt 1285–1486) was titrated with repressor. At 10^{-8} M DNA, nonspecific binding of repressor to the DNA was evidenced by a generalized smear of labeled fragment from uncomplexed to much higher molecular weight material, with no apparent unique complexes (Figure 4A). At high protein concentrations, the visible labeled complexes most likely represented DNA fragments totally saturated with repressor (one repressor covering approximately 20–25 bp). A similar repressor titration at a DNA concentration of 10^{-10} (Figure 4B) showed no discernible shift of the labeled fragment, indicating that repressor did not significantly interact with the DNA at this concentration.

Repressor Binding in the Presence of the Inducer IPTG.[1] To further confirm that nonspecific association of repressor with DNA could be differentiated from its specific binding, both the 207- and 201-bp fragments were titrated with repressor in the presence of inducer (1 mM IPTG). At DNA concentrations of 10^{-8} M, both titrations were similar to the results of Figure 4A, indicating nonspecific association. At DNA concentrations of 10^{-10} M, both titrations resembled the results of Figure 4B, indicating no association of repressor with the DNA. These results were similar to those of previous analyses of the effects of IPTG on repressor binding (Fried & Crothers, 1981; Kim & Kim, 1983): IPTG perturbs specific operator binding without disrupting nonspecific affinity of repressor for DNA. In these gel analyses, repressor did not appreciably associate nonspecifically with DNA at concentrations of 10^{-10} M.

Repressor Binding to Linear Eight-Operator Molecules. Repressor titration of the 292-bp, eight-operator linear fragment, at 10^{-10} M DNA, is shown in Figure 5. Eight unique complexes were observed, with eventual saturation of every binding site. Several criteria established that these bands represented discrete, specific binding interactions. First, the titrations were performed below the nonspecific DNA affinity constant for repressor in this system. An identical pattern of eight unique complexes with eventual saturation was also obtained at a DNA concentration of 10^{-8} M. Second, the titrations resulted in a predictable number of complexes (eight), rather than the 11–12 that would be expected if repressor nonspecifically associated with every 20–25 bp of DNA (Figure 3A). Third, repressor titrations of the 292-bp fragment in the presence of IPTG, at both 10^{-10} M DNA concentrations, were identical with those obtained with the 207- and 201-bp molecules in the presence of IPTG (analogous to Figure 4). At a DNA concentration of 10^{-8} M, IPTG reduced the discrete pattern to a nonspecific smear, and at 10^{-10} M DNA, repressor did not significantly associate with the po-

[1] Abbreviation: IPTG, isopropyl β-D-thiogalactoside.

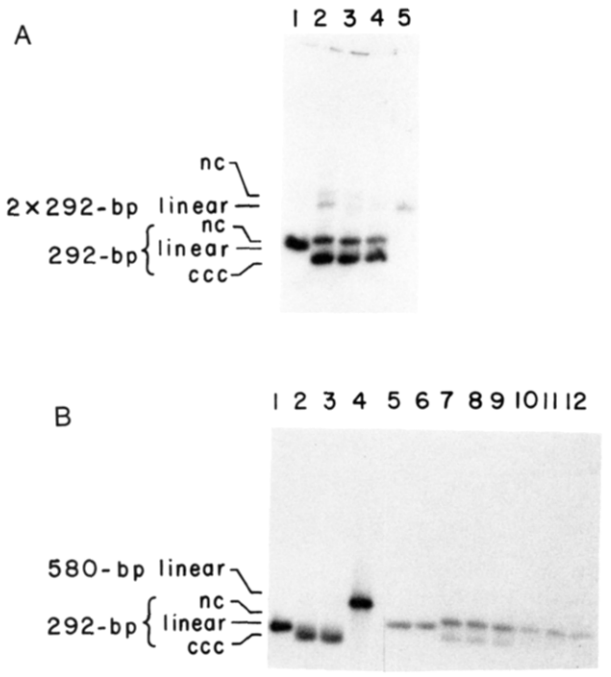

FIGURE 2: Analysis of ligation products of 292-bp polyoperator fragments with 2% (w/v) agarose–7 μg/mL chloroquine gels (Tris–acetate buffer). (A) Effect of DNA concentration on ligation of 292-bp polyoperator. Lane 1, linear 292-bp fragment; lanes 2–4, ligations of 292-bp fragment at 100, 50, and 25 ng/mL DNA, respectively; lane 5, 580-bp linear polyoperator fragment. (B) Production of covalently closed and nicked circular polyoperator molecules. Lane 1, linear 292-bp fragment; lanes 2 and 3, ligations of 292-bp fragment at 25 and 12.5 ng/mL DNA, respectively; lane 4, linear 580-bp polyoperator fragment; lane 5, linear 292-bp fragments labeled by phosphorylation with [γ-^{32}P]ATP without unlabeled ATP; lane 6, linear 292-bp molecule labeled as for lane 5 but with an unlabeled ATP chase; lanes 7–9, covalently closed circular and nicked circular DNA resulting from ligation of polyoperator fragments at 25, 10, and 5 ng/mL DNA, respectively; lanes 10–12, *Eco*RI nicking of covalently closed circular polyoperator molecules in the presence of 100 mM ethidium bromide for 30, 60, and 120 min, respectively.

lyoperator fragment. Lastly, the mobilities of complexes DR_1 through DR_8, which decreased logarithmically, were consistent with the sequential addition of repressor tetramers.

Similar results were obtained with the 580-bp, 16-operator molecule: sixteen unique repressor–DNA complexes were resolvable on agarose gels. However, technical problems associated with analysis of these larger molecules (both 580- and 1156-bp polyoperator molecules), such as the unmanageable gels necessary to resolve the complexes, led us to concentrate on the 292-bp, eight-operator fragment.

Repressor Binding to Circular Eight-Operator Molecules. Repressor titration of covalently closed circular eight-operator molecules (Figure 6A) differed from an identical titration of linear eight-operator molecules (Figure 6B). The eight sites in the covalently closed circular molecule were not saturated by repressor, even at repressor:operator ratios of 5:1 (repressor:fragment ratios of 40:1). At the highest concentrations of repressor tested, the complexes DR_2 and DR_3 still appeared to be the predominant species. The small size of these polyoperator circles suggested several possible explanations for the unexpected nonsaturable binding pattern. The simplest, trivial, explanation is that repressor was excluded from binding adjacent operator sites because of steric constraints due to the size of the polyoperator circle. Another explanation is that some of the operator sites became energetically unavailable for specific binding due to the torsional energy transferred into them by repressors binding at nearby sites, an argument stemming from observations that specific repressor binding perturbs the DNA structure (Kim & Kim, 1983).

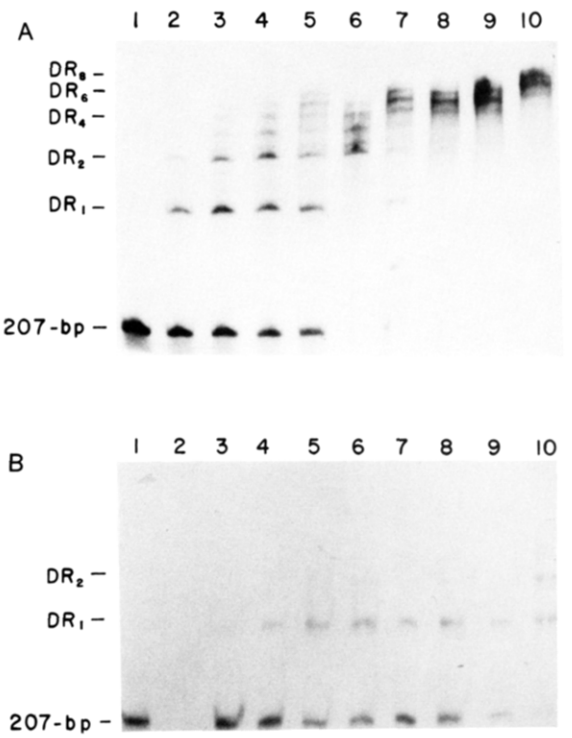

FIGURE 3: Gel shift analysis of *lac* repressor binding to a 207-bp *lacPO* fragment at (A) 10^{-8} M and (B) 10^{-10} M DNA concentration. [^{33}P]DNA and repressor were equilibrated in Tris–borate buffer at 25 °C and electrophoresed in 0.75-mm 5% (4.94:0.06) polyacrylamide gels. Molar ratios of active repressor to DNA fragments were as follows: lane 1, 0; lane 2, 0.5; lane 3, 1.0; lane 4, 1.5; lane 5, 2.0; lane 6, 2.5; lane 7, 3.0; lane 8, 4.0; lane 9, 5.0; lane 10, 6.0. The complexes containing increasing numbers of repressors are indicated as DR_1, DR_2, etc.

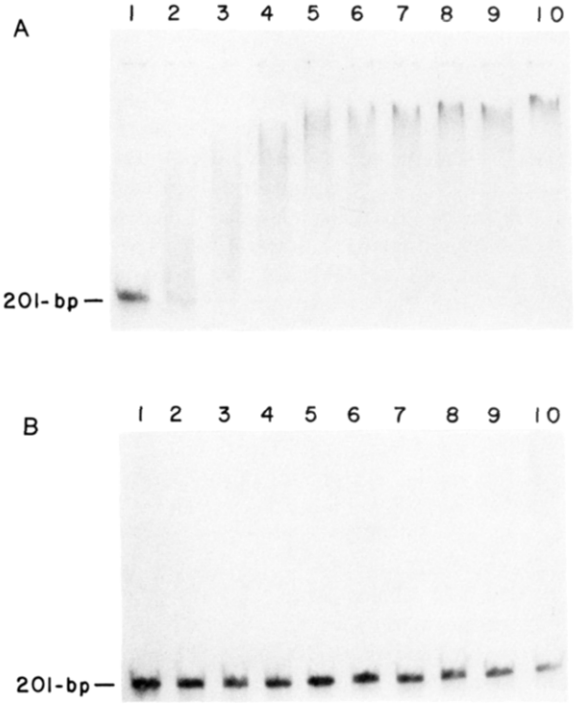

FIGURE 4: Gel shift analysis of *lac* repressor association with a 201-bp nonoperator DNA fragment at (A) 10^{-8} M and (B) 10^{-10} M DNA concentration, analogous to Figure 3. Molar ratios of repressor to DNA fragments were as follows: lane 1, 0; lane 2, 0.5; lane 3, 1.0; lane 4, 1.5; lane 5, 2.0; lane 6, 2.5; lane 7, 3.0; lane 8, 4.0; lane 9, 5.0; lane 10, 6.0.

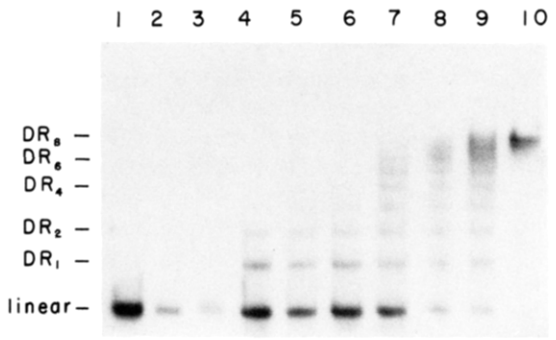

FIGURE 5: Gel shift analysis of *lac* repressor binding to a 292-bp, eight-operator fragment at 10^{-10} M DNA concentration. [^{32}P]DNA and repressor were equilibrated in Tris–acetate buffer at 25 °C and electrophoresed in 1.5-mm 1.4% (w/v) agarose gels as detailed under Experimental Procedures. Molar ratios of repressor to DNA fragments were as follows: lane 1, 0; lane 2, 0.44; lane 3, 0.60; lane 4, 0.75; lane 5, 1.50; lane 6, 3.0; lane 7, 4.5; lane 8, 6.0; lane 9, 7.5; lane 10, 15.0.

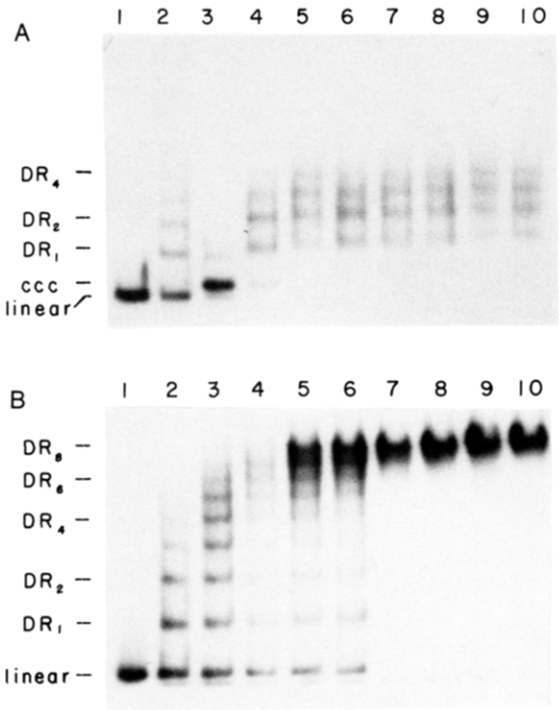

FIGURE 6: Gel shift analysis of *lac* repressor binding to covalently closed circular versus linear 292-bp polyoperator molecules. DNA fragments at 10^{-10} M were equilibrated with repressor in Tris–acetate buffer at 25 °C and complexes electrophoresed on 1.4% (w/v) agarose gels. (A) Lane 1, linear 292-bp DNA; lane 2, linear 292-bp DNA with 1.8 repressors/fragment. Lanes 3–10 contain covalently closed circular molecules incubated with the following molar ratios of repressor: lane 3, 0; lane 4, 1.8; lane 5, 2.2; lane 6, 4.4; lane 7, 8.9; lane 8, 13.3; lane 9, 22.2; lane 10, 44.4. (B) The linear 292-bp polyoperator molecule was incubated with increasing molar ratios of repressor: lane 1, 0; lane 2, 1.3; lane 3, 1.8; lane 4, 2.2; lane 5, 4.4; lane 6, 8.9; lane 7, 13.3; lane 8, 17.8; lane 9, 22.2; lane 10, 44.4.

If the disruption of repressor binding to adjacent sites results from a structural perturbation retained by the continuity of the two DNA strands, then the nicking of one strand should relieve the constraint. Conversely, if the inhibition of repressor binding to all available operator sites results from a steric constraint (due to the small fragment size), then relieving the constraint of the continuous strands by nicking should not change the binding pattern. Repressor titrations of nicked circular eight-operator molecules (Figure 7) showed that at repressor:operator ratios of 22:1 (half the maximal ratio used with the covalently closed molecules) the nicked polyoperator

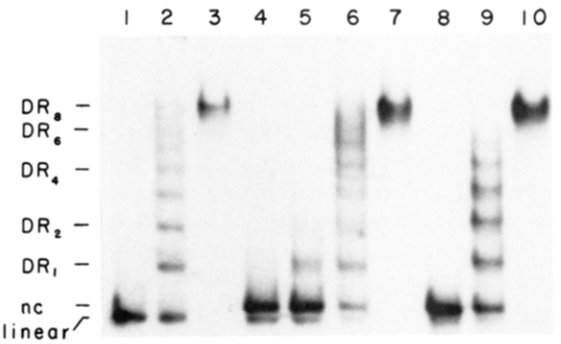

FIGURE 7: Gel shift analysis of *lac* repressor binding to linear and nicked circular 292-bp polyoperator molecules, analogous to Figure 6. Lanes 1–3, linear 292-bp molecules; lanes 4–7, nicked circles purified from agarose gels; lanes 8–10, nicked circles generated by *Eco*RI nicking. The molar ratios of repressor to fragment were as follows: lane 1, 0; lane 2, 2.2; lane 3, 22.2; lane 4, 0; lane 5, 2.2; lane 6, 8.9; lane 7, 22.2; lane 8, 0; lane 9, 8.9; lane 10, 22.2.

circles were saturated with repressor (DR_8 complexes). The restricted number of repressor binding sites in covalently closed polyoperator molecules was alleviated by simply nicking one strand of the duplex.

Discussion

Our results show qualitatively that *lac* repressor saturates multiple binding sites on both linear and nicked circular polyoperator molecules but not on covalently closed circular molecules. We did observe that binding at neighboring sites of linear and nicked circular molecules was partially diminished at less than saturating repressor concentrations (Figures 5 and 7). For example, at protein:DNA ratios of 4:1 where random stoichiometric binding predicts a distribution around DR_4, the complexes actually detected ranged from D (unoccupied) to DR_8 (fully occupied), with the predominant complexes being those containing fewer than four repressors. This pattern is suggestive either of nonstoichiometry or possibly of site exclusion, whereby binding at one site partially inhibits binding at additional sites. However, for both the linear and nicked circular molecules, all eight sites were saturated at high protein concentrations. Our in vitro studies found no evidence of loop formation, but then, the high molar ratios of protein to DNA (1:1 to 40:1) used to investigate multiple repressor binding would not have favored loop formation (Kramer et al., 1987).

Ligation closure of DNA duplexes into covalent circles results in families of molecules differing in topoisomeric states (Depew & Wang, 1975). Because of its small size, the eight-operator fragment was expected to exist in predominantly one topoisomer (Horowitz & Wang, 1984; Shore & Baldwin, 1983; Kramer et al., 1988). Topoisomers thus might only partially explain the distribution of complexes observed with the covalently closed circular molecules at high repressor concentrations, which did not show saturation to a maximum single complex (Figure 6A, lanes 8–10). Specific repressor binding to operator alters the structural conformation of the operator site by either unwinding or bending (Kramer et al., 1987, 1988; Wang et al., 1974; Kim & Kim, 1983; Zwieb et al., 1989). If binding unwinds (or overwinds) the DNA helix, then molecules that are already under structural tension by being closed in that state might not successfully accommodate a saturating number of repressor molecules, even at high levels of protein. Perturbation of the helix by 55° unwinding per specific interaction cannot, however, totally account for the results seen here. The removal of 360° of rotation, equivalent to the difference between the two closest topoisomeric states, would require binding of six to seven repressors. If only two

topoisomers were available to the 292-bp fragment, one or the other of the two states should conceivably have bound six to seven repressors in going to the second state. No complexes with that number of repressor bound were observed with the covalently closed molecules. Alternatively, the covalent DNA duplexes may be unable to adopt certain structural conformations, or access to their binding sites may be sterically restricted differently from those of the nicked circular molecules. It is presently unclear how the exclusion of multiple repressors manifested with covalently closed circular structures was relieved simply by nicking one DNA strand.

The natural *lac* operator is flanked by two weak repressor binding sites, the *lacI-P* and *lacZ* pseudooperators. A physiological role for these pseudooperators is suggested by the fact that repression of the operon is decreased following destruction of the *lacZ* pseudooperator site (Eismann et al., 1987). Conversely, repression of artificial operons is increased following insertion of a second operator upstream or downstream of the transcriptional start site (Herrin & Bennett, 1984; Mossing & Record, 1986; Besse et al., 1986). Methylation studies showing cooperative protection by repressor of the natural operator and *lacZ* pseudooperator (Flashner & Gralla, 1988) also indicate that multiple protein binding sites in close proximity can affect the occupancy of each other. The functional consequences of a conformational change in the natural *lac* operon, generated by specific repressor binding to the operator, have not been investigated in detail. However, the position of the natural operator at +1 relative to the start of transcription may reflect a role for an induced conformational change on the interaction of repressor and RNA polymerase with this region.

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