How *lac* repressor recognizes *lac* operator*

(5 methyl of thymine/2 amino of guanine/major groove/minor groove/one side of DNA)

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ABSTRACT Nucleotide analogs were substituted for unmodified nucleotides at specific sites in the *lac* operator sequence by a combination of chemical and enzymatic procedures. The nitrocellulose filter assay was used to study the interactions of these modified operators with wild-type (SQ) and tight-binding (QX86) *lac* repressors. These studies implicate directly the 5 methyl of thymine and the 2 amino of guanine as important operator-repressor contact sites. Furthermore, when these findings are combined with published results from other laboratories, a model for the *lac* operator-*lac* repressor interaction can be derived. Two important postulates follow from this model. (i) The repressor interacts at specific and defined sites with the N7 of guanine, the 5 methyl of thymine, the 2 amino of guanine, and the central major groove of the operator. (ii) The repressor binds to one side of the operator.

DNA-protein interactions are of fundamental importance in a large variety of molecular processes, yet are not well understood biochemically (1, 2). We are currently studying the *lac* operator-repressor system in order to determine the mechanisms by which proteins recognize and interact with specific DNA sequences.

The *lac* repressor protein binds tightly to the *lac* operator, a unique sequence in the *Escherichia coli* chromosome. An examination of operator-constitutive (o^c) mutants has identified eight base pairs that are involved in binding to repressor (3, 4). Gilbert *et al.* (5) have shown that the binding of repressor to operator specifically protects four guanines and three adenines against methylation with dimethyl sulfate. At the same time, the methylation of two guanines and one adenine is enhanced. (Dimethyl sulfate methylates double-stranded DNA at the N7 of guanine and the N3 of adenine exposed, respectively, in the major and minor grooves.) Crosslinking experiments have identified thymine residues that contact repressor in the major groove (6). Thus the *lac* repressor appears to interact with operator DNA in both the major and minor grooves. All these sets of data are shown in Fig. 1.

One primary objective of research from this laboratory is to decipher those elements of the *lac* operator bases that stabilize the repressor-operator (RO) complex. The approach outlined in this paper involves insertion of nucleic acid base analogs at specific sites in the DNA, followed by analysis of how these analogs affect the stability of the RO interaction. These results, when used in conjunction with the above sets of data, have allowed us to deduce several *lac* operator sites that are recognized by *lac* repressor.

MATERIALS AND METHODS

Syntheses of unmodified and various modified *lac* operator DNAs have either been described (7-11) or remain to be published. Nitrocellulose filter binding assays were performed as

described previously (9, 12, 13). Wild-type (SQ) repressor was purified by a published procedure (9). Tight-binding (QX86) repressor was provided by J. Sadler.

RESULTS

The 26 operator duplexes listed in Table 1 were prepared by a combination of chemical and enzymatic methods. These operators include wild-type DNA (duplexes I and II) and 24 DNAs that contain site-specific base modifications. After each synthetic step, deoxyoligonucleotides were purified and monitored for homogeneity by column procedures and by gel electrophoresis (9-12).

The effects of nucleotide alterations on the binding of operator to SQ and QX86 repressors were determined by using the nitrocellulose filter assay (14). Results (12) with unmodified duplexes I and II were used as standards. Whenever possible, rates of dissociation (expressed as half-lives) for RO complexes rather than equilibrium constants were measured. However, the RO complex between duplex I (15 C-H) and SQ repressor had a half-life too short to measure directly. Therefore duplex I (15 C-H) was analyzed by the equilibrium competition method (13). The results of these binding experiments are shown in Table 1.

DISCUSSION

Data presently available suggest that *lac* repressor recognizes double-stranded, base-paired *lac* operator (2, 15, 16). However, conformations of *lac* operator and *lac* repressor in the RO complex are not known (A DNA, B DNA, or variations of these basic types). Specificity of recognition could occur at the edges of stacked bases accessible in the major and minor grooves. Theoretical discussions of potential recognition processes have previously been developed (17-19). A protein capable of probing the major groove would be able to contact substituents on the 4, 5, and 6 positions of pyrimidines and the 6, 7, and 8 positions of purines. Substituents at position 2 on pyrimidines and positions 2 and 3 on purines would be accessible in the minor groove.

Binding experiments were performed to determine the dissociation half-lives or equilibrium constants for RO complexes containing modified nucleotides. The ratios of half-lives for RO complexes (modified compared to unmodified operators) were used to compute changes in binding free energy caused by these modifications. Table 1 summarizes the free energy changes. These calculations are possible because association rate constants are unaffected by nucleotide alterations (3) and are largely electrostatically controlled (12). When these results are considered in conjunction with earlier studies on O^c mutants (3, 4),

Abbreviations: O^c , operator-constitutive; RO, repressor-operator.

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FIG. 1. Summation of the *lac* operator-*lac* repressor interactions. The top part shows the *lac* operator sequence. The heavy lines above and below the sequence delineate 2-fold symmetric regions. The dyad axis is indicated by the arrow. Below the sequence is a summation of relevant experimental data. Methylation data summarizes repressor protection experiments with dimethyl sulfate (5). Methylation protection (-) or enhancement (+) on N7 of guanine or N3 of adenine is indicated immediately below the appropriate base pair. Sequence changes leading to the *O_c* phenotype (4) are also shown. Finally, sites crosslinked with repressor (+) in the presence of UV light are listed. The crosslinking occurs in the major groove when 5-bromouracil replaces thymine (6). Duplex I is sequence 1-26 and duplex II is 6-26.

operator methylation patterns (5), and RO crosslinking experiments (6), several *lac* operator recognition sites can be predicted. These sites are discussed in the following paragraphs and are summarized in Table 2 and Fig. 2. Many of these predictions are based on the model of Seeman *et al.* (19) concerning sequence-specific recognition of DNA by proteins. The terms "central major groove," "outer major groove," "central minor groove," and "outer minor groove" are used as defined by Seeman *et al.* (19).

Some postulated recognition sites are at least partially dependent on a negative result: the lack of *O_c* mutations at a specific locus. This is unavoidable at the present time. Perhaps some of the conclusions outlined below will therefore have to be revised if additional major *O_c* mutants are found.

Operator Sites 6, 7, 25, and 26. There are no known *O_c* mutations at positions 6, 7, 25, and 26. This observation implies that either major interactions do not occur at these positions or the interactions must be confined to functional groups unaltered electronically by transitions and transversions (19). Unaltered sites are found in the outer minor groove (2 keto of thymine and N3 of adenine). However, repressor binding does not change the operator alkylation pattern with dimethyl sulfate (5), indicating that the adenine side of the minor groove is free of repressor. Thus the 2 keto of thymine remains as the only potential major contact site. Several different experiments suggest that *lac* repressor interacts to some extent with these base pairs in the major groove. Crosslinking occurs at all four sites, indicating that repressor lies close to the 5 methyl of thymine. Replacing these methyls with hydrogen weakens the binding to repressor [compare II, II (6,7 A-U), and II (6,7 A-U; 25,26 U-A)]. Also, duplex I (6,7,13 A-U) forms a less stable complex than I (13 A-U) with QX86 repressor. Insertion of a less hydrophobic bromine atom for a methyl group usually destabilizes these complexes. This data suggests a weak hydrophobic contact in the major groove between the 5 methyl of thymine and the repressor. Subtle DNA conformation changes induced by 5-bromouracil or uracil rather than weak hydrophobic contacts could also explain the altered stability of the RO complexes. Because insertion of 5-bromouracil renders the stability of these RO complexes completely independent of ionic strength between 0.05 and 0.20 M (10), such a possibility cannot be excluded by these experiments. Bahl *et al.* (20) have concluded that all the nucleotides essential for the RO interaction are

within the sequence 8-24. However, our results suggest that minor but specific interactions occur outside this sequence.

Operator Sites 8, 9, 23, and 24. Crosslinking experiments have shown that these positions are covered by repressor in the major groove (6). The adenines at 8, 9, and 24 are protected by repressor against methylation (5), implying that repressor covers the minor groove at these positions. If an important stabilizing RO interaction occurs, then the lack of *O_c* mutants indicates that only the outer minor groove (N3 of adenine, 2 keto of thymine) is capable of specific binding to repressor (19). However, repressor fails to protect adenine 23 against alkylation with dimethyl sulfate. Thus the N3 of adenine 23 is not involved in repressor recognition. Substitution of 5-bromouracil for thymine does not affect repressor binding at the symmetrically related sites 8 and 24. Substitution of 5-bromouracil at either 9 or 23 (also symmetrically related) results in an identical increase in the stability of the operator-QX86 repressor complex. Opposite effects occur with SQ repressor: 5-bromouracil substitution at 9 increases RO complex stability, whereas substitution at 23 decreases stability. If direct contact between repressor and 5-bromouracil occurs in the major groove, enhanced stability relative to thymine is postulated as a dipole or induced dipole interaction, whereas decreased stability is postulated as a hydrophobic contact (10). Thus 5-bromouracils at 9 and 23 appear to be near ionic or polar QX86 repressor groups. With SQ repressor, however, only 9 appears to be in this type of environment. 5-Bromouracil at 23 might be in a hydrophobic SQ repressor region. Therefore as at 6, 7, 25, and 26, the thymine methyl group at 23 might be interacting with a hydrophobic SQ repressor region. This must be a weak contact because a major *O_c* mutant has not been detected.

Operator Sites 10 and 22. Considerable evidence suggests that important DNA-protein contacts occur in the major groove at these G-C base pairs. The major groove is implicated because guanine N7 is protected by repressor against methylation (5). The *O_c* mutation (G-C → A-T) weakens repressor binding significantly (3, 4). According to the model of Seeman *et al.* (19), major groove recognition elements consistent with this *O_c* are the 4 amino of cytosine, 6 carbonyl of guanine and, by steric hindrance, the 5 methyl of thymine. The N7 of guanine does not appear to be an interaction site because a G-C to A-T transition retains the N7 on the imidazole ring in the same relative position. Steric hindrance caused by the 5 methyl of thymine

Table 1. Dissociation half-lives and binding free energy changes for 5-bromouracil-, 5-bromocytosine-, hypoxanthine-, and uracil-substituted operator duplexes

Duplex*	SQ repressor		QX 86 repressor	
	$t_{1/2}$, sec	ΔG , kJ/mol [†]	$t_{1/2}$, min	ΔG , kJ/mol [†]
II	38		13	
I	47		21	
II (6 A-BrU)	26	(-) 0.9	12	0
II (7 A-BrU)	26	(-) 0.9	13	0
II (8 BrU-A)	36	0	13	0
II (9 BrU-A)	65	(+) 1.3	17	(+) 0.7
I (10 G-BrC) [§]	28	(-) 1.3	10	(-) 1.8
II (11 BrU-A)	72	(+) 1.6	21	(+) 1.2
I (12 G-BrC)	39	(-) 0.5	18	(-) 0.4
I (12 H-C)			35	(+) 1.3
II (13 A-BrU)	30	(-) 0.6	8	(-) 1.2
I (13 A-U)	<10 [†]		1.7	(-) 6.2
I (15 C-H)	<10 [†]	(-) 7.1		
I (18 A-BrU)	64	(+) 0.8	20	0
I (18 A-U)	44	0	14	(-) 1.0
I (19 BrU-A)	73	(+) 1.1	26	(+) 0.5
I (20 A-BrU)	45	0	20	0
I (20 A-U)	39	(-) 0.5	21	0
II (21 A-BrU)	36	0	11	(-) 0.4
II (23 A-BrU)	34	(-) 0.3	17	(+) 0.7
II (24 A-BrU)	35	0	13	0
II (25 BrU-A)	29	(-) 0.7	10	(-) 0.7
II (26 BrU-A)	33	(-) 0.4	11	(-) 0.4
II (6,7 A-U)	25	(-) 1.0	11	(-) 0.4
II (6,7 A-U; 25,26 U-A)	20	(-) 1.6	9	(-) 0.9
I (6,7,13 A-U)	<10 [†]		1.2	(-) 7.1

* Duplex I is sequence 1-26 and duplex II is 6-26 (see Fig. 1). Parentheses following these numerals indicate nucleoside modifications within these duplexes. The position number of a modification site is followed by the base pair at that site. The symbols are: BrU, 5-bromouracil; BrC, 5-bromocytosine; U, uracil; H, hypoxanthine. As an example, duplex I (15 C-H) refers to duplex I in which position 15 has been altered to contain a cytosine-hypoxanthine base pair.

[†] (-) indicates that the modification weakens the RO interaction (i.e., increases ΔG ; (+) indicates that the modification strengthens the RO interaction. Identical reaction conditions including an ionic strength of 0.05 M were used for all experiments.

[‡] The precision of dissociation experiments is $\pm 10\%$. Therefore dissociation half-lives within 10% of unmodified duplexes are given a binding free energy change of 0.

[§] This duplex was not entirely base paired. The sequence d(T-T-C-C-A-C-A) was missing (positions 1-7).

^{||} The dissociation of these RO complexes was complete by 15 sec.

^{||} Calculated from the change in the equilibrium dissociation constant (K_d) for the RO interaction when compared to unmodified duplex I (17-fold).

can also be eliminated as the source of the O^c phenotype. As shown in Table 1, replacement of the 5 hydrogen of cytosine with bromine (1.95 Å compared to 2.0 Å for the methyl group) weakens the RO interaction by only a fraction of the energy observed for the transition [1.3 vs. 6.7 kJ/mol for QX86 repressor (3) at site 10]. Because 5-bromocytosine has not been inserted at site 22, the possibility exists that the 5 methyl is responsible for this O^c mutation. Hydrogen bonding interactions with the 2 amino of guanine could also occur because a G-C to A-T transition would eliminate this contact. However, the evidence indicates that the major groove is covered by repressor at these positions. Therefore major groove interactions through the 4 amino of cytosine or the 6 carbonyl of guanine appear to be the most likely.

Operator Sites 11, 18, 20, and 21. The minor groove is partially free of repressor, because the adenines are accessible to dimethyl sulfate in the presence of repressor (5). The major groove appears covered by repressor. Substitution of 5-bromouracil for thymine at site 11 strengthens the RO interaction by 1.6 and 1.2 kJ/mol with SQ and QX86 repressors, respectively. Repressor crosslinking in the major groove was observed at sites 18, 20, and 21 (6). Alkylation experiments with the pseudo-operator in the β -galactosidase gene suggest that the major groove may be covered at site 18. A G-C base pair at this position is protected by repressor against major groove methylation (6). However, O^c mutations have not been found at these sites. This observation implies that important major groove contacts between operator and repressor do not occur. This conclusion is consistent with the observation that analogs (uracil and 5-bromouracil) altering the major groove do not appreciably weaken the RO interactions.

Operator Site 12. The *lac* repressor appears to recognize the N7 position of guanine. This site is protected by repressor against dimethylsulfate methylation (5). A G-C to T-A transversion weakens the RO interaction 5.0 kJ/mol with QX86 repressor (3, 4). Using arguments presented by Seeman *et al.* (19), we conclude that the interaction must involve the outer major groove (N7 of guanine or H5 of cytosine) or the 2 amino of guanine in the minor groove. As shown in Table 1, substitu-

Table 2. Possible repressor recognition sites in *lac* operator DNA

Positions	Groove utilized*	Recognition elements [†]	
6, 7	M	Thymine	5 methyl
	m	Thymine	2 carbonyl
8, 9	M		
	m	Adenine	N3
		Thymine	2 carbonyl
10	M	Cytosine	4 amino
		Guanine	6 carbonyl
11	M		
12	M	Guanine	N7
13	M	Thymine	5 methyl
14	m	Guanine	2 amino
15	m	Guanine	2 amino
16	M	Cytosine	4 amino
	m	Guanine	2 amino
17	M		
18	M		
19	M	Adenine	6 amino
		Thymine	4 carbonyl
20	M		
21	M		
22	M	Cytosine	4 amino
		Guanine	6 carbonyl
23	M	Thymine	5 methyl [‡]
	m	Thymine	2 carbonyl
24	M		
	m	Adenine	N3
		Thymine	2 carbonyl
25, 26	M	Thymine	5 methyl
	m	Thymine	2 carbonyl

* Refers to the groove most likely covered by repressor protein regardless of whether a specific interaction occurs. M represents the major groove and the m the minor groove.

[†] All recognition elements that cannot be eliminated are included. In some cases, for example N3 of adenine or 2 carbonyl of thymine, no experimental data exclude the sites. The model does not imply that all the listed sites are critical for repressor interaction. Some will undoubtedly be eliminated by additional experiments.

[‡] SQ repressor only.

FIG. 2. Postulated location of the repressor relative to the operator and location of major DNA interaction sites. The general, very schematic, location of the repressor is shown by the solid, white line. White arrows extending from this line point to the DNA functional groups that interact strongly with repressor. Most of these functional groups are visible in this view (sites 10, 12, 13, 15, 16, 22). However two sites (14, 19) are slightly hidden but readily visible when viewed from above. The black arrow marks the dyad axis.

tion of 5-bromocytosine for cytosine weakens the RO interaction only slightly. Therefore repressor does not contact operator on the cytosine side of the major groove. Replacement of guanine with hypoxanthine has no destabilizing effect on the RO complex. This result eliminates the 2 amino as a contact site. Therefore the imidazole N7 of guanine is implicated as the repressor recognition site.

Operator Site 13. The *lac* repressor recognizes the 5 methyl of thymine. Replacement with uracil weakens the RO interaction by 6.2 kJ/mol with QX86 repressor. This increase in binding free energy is comparable to the change observed with QX86 repressor and the O^c mutation at site 13 [7.5 kJ/mol (3)]. The importance of the 5 methyl can also be demonstrated by comparing the repressor binding of duplexes II (6,7 A-U) and I (6,7,13 A-U). Replacement with uracil at sites 6 and 7 results in only a slight weakening of the RO interaction, whereas an additional uracil at site 13 dramatically increases the binding free energy. Insertion of 5-bromouracil for thymine also reduces the stability of the RO complex. Both effects can be explained by an important hydrophobic contact between *lac* repressor and the 5 methyl of thymine (Fig. 1). The enhanced methylation of adenine by dimethyl sulfate (5) could be the result of a hydrophobic repressor region close to the minor groove. As previously suggested (5), such a region might increase the local concentration of dimethyl sulfate. Therefore, in addition to probing the major groove and interacting with the 5 methyl of thymine, hydrophobic repressor regions probably lie adjacent to the minor groove at site 13.

Operator Site 14. Data presently available indicate that the repressor recognizes this G-C base pair at the 2 amino group. The O^c mutation (G-C to T-A) changes the recognition elements in the outer major (N7 of guanine) and central minor (2 amino of guanine) grooves (19). Yet methylation of guanine N7 is enhanced when repressor is bound, indicating that repressor does not contact this site. The enhancement could be due to the

hydrophobic repressor region that is important at site 13 in the major groove. This hydrophobic region could cause an increase in the local dimethyl sulfate concentration, leading to an increase in reactivity at site 14 (5). This leaves the 2 amino of guanine as the most likely candidate for repressor recognition.

Operator Site 15. The *lac* repressor interacts with the 2 amino of guanine. Substitution of hypoxanthine for guanine rendered the RO complex completely unstable to nitrocellulose filters. However, a 17-fold increase in the RO equilibrium dissociation constant (K_d) can be measured (data not shown) by the equilibrium competition method (13). This corresponds to a 7.1 kJ/mol increase in the binding free energy (Table 1). The C-G to T-A transition (3, 4) weakens the RO interaction by 7.9 kJ/mol for QX86 repressor. Therefore removal of the 2 amino of guanine accounts for most of the change in binding free energy.

Operator Site 16. The G-C to A-T transition weakens repressor binding more than any characterized single-site O^c mutation [the free energy of binding to QX86 repressor is increased by 8.4 kJ/mol (3, 4)]. Possible candidates for interaction with repressor are the 4 amino of cytosine, the 6 carbonyl of guanine, and the 2 amino of guanine. However, guanine N7 is not protected by repressor against dimethyl sulfate alkylation. Therefore at least the guanine portion of the major groove is accessible to solvent and not covered by repressor. Thus the most likely candidates for repressor interactions are the 2 amino of guanine and the 4 amino of cytosine.

Operator Site 17. There appear to be no major contacts between repressor and operator at this position. The only evidence is the lack of O^c mutants. This negative result implies that the only possible specific recognition sites are in the outer minor groove. However, the pseudo-operator of the β -galactosidase gene has an A-T rather than G-C base pair at position 17 and no change in alkylation with dimethyl sulfate is observed in the presence of repressor (5). Consequently the minor groove ap-

pears free of repressor. Conversely, the N7 of guanine is protected by repressor against alkylation with dimethyl sulfate (5). Therefore the repressor appears to block access to at least the guanine side of the major groove without actually being involved in favorable interactions.

Operator Site 19. Repressor appears to interact in the major groove. The minor groove is free of repressor and accessible to dimethylsulfate (5). The T-A to C-G transition increases the binding free energy by 2.5 kJ/mol with QX86 repressor (3, 4). Major groove positions altered by the O^c mutation are the 5 methyl and 4 carbonyl of thymine and the 6 amino of adenine. Insertion of 5-bromouracil for thymine stabilizes the RO complex. This result implies that the 5 methyl is not critical as a hydrophobic contact site (compare effects with site 13). Therefore the operator is recognized by repressor through the 6 amino of adenine and/or the 4 carbonyl of thymine.

The Model. A model for the RO interaction follows from these considerations (Table 2 and Fig. 2). Along the operator from left to right (Fig. 2), repressor covers the operator in the major and minor grooves at positions 6–9, the major groove at 10–13, and the minor groove at 14–16. Positions 16–22 are covered in the major groove and 23–26 in both grooves. The outer DNA boundaries of the RO interaction are not defined by these experiments. This spacing would allow repressor to contact the operator from one side and interact with both grooves. Such a model suggests that the repressor does not lie parallel to the helix axis of B form operator but crosses this axis at an angle (10–20°). This same conclusion was derived from the results of phosphate alkylation experiments (W. Gilbert, personal communication).

The binding of repressor is known to unwind the operator by approximately 90° (21). How the unwinding relates to this model cannot be determined from the results presented here. Furthermore, the model does not include any intercalative interactions that may involve repressor side chains.

Additionally, these results reveal operator contact sites that stabilize the RO complex. Postulated strong contacts are shown in Fig. 2. These are the 5 methyl of thymine (position 13), the 2 amino of guanine (positions 14, 15, 16), the N7 of guanine (position 12), and the central major groove functional groups at positions 10, 19, and 22.[†] The 4 amino of cytosine at 16 cannot be excluded as a potential, major interaction site. However, this group protrudes into the major groove on the side that, through visual inspection of the model, is not covered by repressor. Therefore the 4 amino would appear to be a highly unlikely contact site. Weak contacts have been detected but are not shown in Figure 2. These involve the 5 methyl of thymine at

6, 7, 23, 25, and 26. Indirect arguments can be made to implicate the outer minor groove at 8, 9, 23, and 24 and the 2 keto of thymine at 6, 7, 25, and 26. Additional base substitution experiments can be used to test these hypotheses.

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[†] Recent experiments with additional uracil analogs and a complete set of hypoxanthine analogs are in complete agreement with the proposed model.