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Sites of contact between λ operators and λ repressor*

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ABSTRACT

DNA bearing λ operator sequences was methylated by dimethyl sulfate (DMS) in the presence or absence of λ repressor. Under the experimental conditions, DMS methylates only the purine residues. The presence of λ repressor affects only the methylation of certain G residues in the operators. Repressor blocks the methylation of certain G's and enhances the methylation of other G's. Since the reactive ring-nitrogen of G lies in the major groove of double-stranded DNA, and the reactive ring-nitrogen of A lies in the minor groove, the above results imply that the repressor makes contacts in the major groove of the helix. The repressor effect on G-methylation is sharply confined to the three 17 base pair units within each λ operator previously proposed as the repressor-binding sites.

INTRODUCTION

The λ repressor, a protein encoded by the cI gene of bacteriophage λ, binds to two operators, termed O_L and O_R, on λ DNA. The interaction between repressor and the operators results in both positive and negative regulation of gene expression. Thus, repressor bound to O_L and O_R blocks the transcription of two separate genes required for lytic growth. In addition, repressor bound to O_R may stimulate or repress transcription of the cI gene itself. Our current understanding of these effects, as described by Ptashne et al. (1), is based on the finding that each operator contains more than one site that independently binds repressor. The complete nucleotide sequence of each operator has now been established (1,2,3). Within each operator, we have identifed three sets of sequences which we believe are specifically recognized by the repressor. These sites, which are similar but not identical in sequence, are 17 base pairs long and are separated from one another by AT-rich spacers 3 to 7 base pairs long (1). All these sequences show partial twofold rotational symmetry, with the axis

passing through the ninth base pair of each sequence. The three hypothetical sites within the left operator (O_L) are termed O_L1, O_L2, and O_L3, and in the right operator (O_R), they are termed O_R1, O_R2, and O_R3. Mutations that reduce the affinity of DNA for repressor have been located in sites O_R1, O_R2, O_L1, and O_L2; mutations in the spacer regions affect the action of RNA polymerase but have no effect on repressor binding. The results of experiments in which operators were exposed to the action of nuclease in the presence of repressor as well as other considerations suggest that repressor binds first to O_L1 (or O_R1) and then sequentially fills the remaining sites. Individual 17 base pair sequences have neither been synthesized nor isolated and therefore we do not have direct confirmation of the role of these sequences in repressor recognition.

Gilbert *et al.* (4) have described how methylation by dimethyl sulfate can be used as a chemical probe to reveal the bases on DNA that are specifically contacted by a bound protein. They used this method to reveal the bases in the *lac* operator covered by the *lac* repressor. We have previously used this method to determine the bases recognized by an unusual restriction endonuclease (5). In this paper we describe experiments that show that repressor makes specific contacts with bases within the hypothesized repressor binding sites but not with bases in the spacers nor with bases in the regions surrounding the operators. Moreover, our results support our previous finding that repressor binds with the highest affinity to the terminal sites O_L1 and O_R1. Finally our results suggest that λ repressor makes close contacts with DNA predominantly if not solely in the major groove of the DNA helix.

MATERIALS AND METHODS

Reagents and Buffers: All chemicals were reagent grade. γ-^{32}P-ATP was prepared as described (6). Methylation buffer: 50 mM Sodium Cacodylate (pH 8.0), 10 mM $MgCl_2$. Electrophoresis buffer: 45 mM Tris-borate (pH 8.3), 1.25 mM EDTA.

Enzymes and Restriction Fragments: The restriction endonuclease HhaI (from *Haemophilus haemolyticus*) was a gift from Andrea Jeffrey. The other restriction enzymes used in preparing

the various restriction fragments were prepared as given earlier (2,5,6). The restriction fragments Alu 180, Hae 790, Hin 320, Hin 375, and Hin 550 were prepared according to previously published methods (2,6).

Selective End Labelling of Restriction Fragments: The various restriction fragments used in the experiments to be described were prepared labelled at one or the other end according to the strategy outlined in Figure 1b and Table 1.

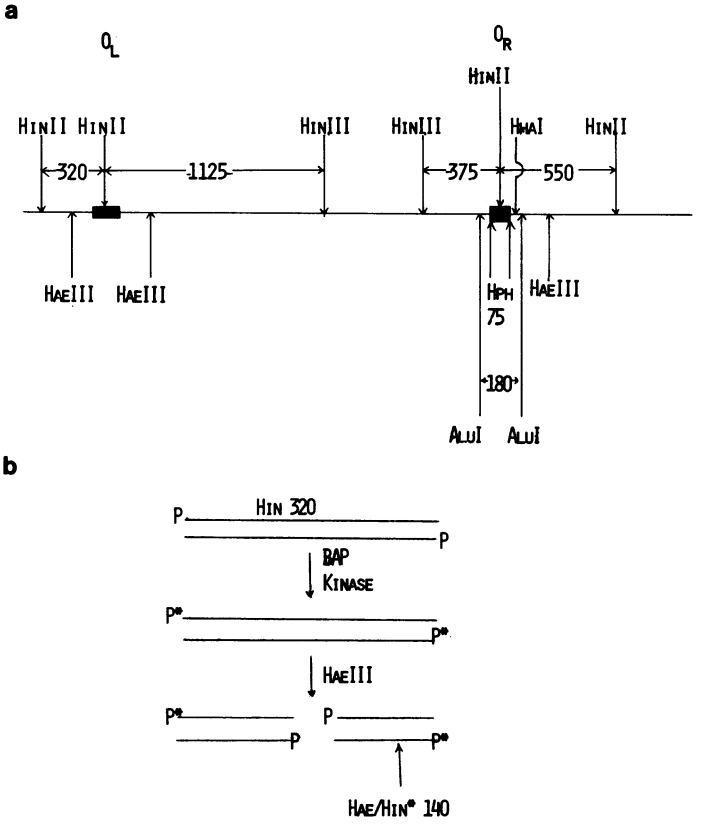

Figure 1

a. A diagrammatic representation of a part of the phage λ genome, showing sites of cleavage by various restriction endonucleases in and around the two λ operators. (Only those sites necessary to the understanding of this paper are shown.) The approximate sizes of some DNA fragments generated by the restriction enzymes are indicated in terms of number of base pairs.

b. A diagrammatic representation of the strategy used for selectively labelling one or the other end of a DNA fragment. BAP is bacterial alkaline phosphatase and kinase is T_4 polynucleotide kinase.

The labelling procedures, which involved dephosphorylation of 5' ends with phosphatase and subsequent rephosphorylation with ^{32}P by T_4 polynucleotide kinase, have been described in detail elsewhere (2).

Table 1. Preparation of Operator-Containing Restriction Fragments Specifically Labelled at One End

(a) End-Labelled Primary Restriction Fragment	(b) Restriction Enzyme Used for Secondary Cleavage	(c) Secondary Fragment Generated	(d) Operator Sequence Contained at Labelled End
Hin 320	HaeIII	Hae/Hin*140	O_L1
Hin 1125	HaeIII	Hae/Hin*190	O_L2+O_L3
Hin 375	AluI	Alu/Hin* 85	O_R2+O_R3
Hin 550	HaeIII	Hae/Hin*135	O_R1
Hph 75	HindII	Hph/Hin* 45	O_R2+O_R3
		Hph/Hin* 30	O_R1
Alu 180	HhaI	Alu*/Hha 160	$O_R1+O_R2+O_R3$

Methylation Procedure: The appropriate labelled operator-containing restriction fragment (0.1 to 1.0 pmol) plus 25 µg of carrier DNA (unlabelled sonicated calf thymus DNA) were incubated with various amounts of repressor (electrophoretic purity greater than 99%, a gift from Robert Sauer) in 0.5 ml of methylation buffer for 5 minutes at 0°. Dimethyl sulfate was added to a final concentration of 25 mM and the reaction allowed to proceed for 15-60 minutes at 20°. The reaction was terminated by adding 2-mercaptoethanol to 0.25 M and all DNA was recovered by precipitation with ethanol (0.1 vol of 20% Sodium Acetate, 50 µg of carrier tRNA and 3 vol of 95% ethanol). The pellet was washed once with 95% ethanol and dried under vacuum.

G-Specific Cleavage of Methylated DNA: The dried pellet after the methylation procedure was dissolved in 20 µl of 1.0 M aqueous piperidine and the solution heated in a sealed capillary for 30 minutes at 90°. Excess piperidine was removed by lyophilization and the residue dissolved in 20 µl of 0.1 N NaOH. 25 µl of a dye mix (0.05% each of xylene cyanol and brom phenol blue in 10 M urea) were mixed with the DNA solution, which was then loaded on gel for fractionation.

Partly A-Specific Cleavage of Methylated DNA: The dried DNA pellet at the end of the methylation procedure was dissolved in 50 µl of H_2O. 25 µg of carrier DNA were added and all DNA in the solution was precipitated by adding 1.0 ml of cold 0.2 M perchloric acid and allowing the contents to stand for one hour at 0°. The precipitate was spun down and the pellet was redissolved in 0.25 ml of H_2O. The DNA was recovered by reprecipitation with ethanol followed by a 95% ethanol wash. The pellet was dissolved in 20 µl of 0.2 N KOH and the solution heated in a sealed capillary at 90° for 15 minutes, mixed with the dye mix and loaded on the gel.

Gel Fractionation of DNA Degradation Products: The degraded DNA from the above reactions was fractionated on polyacrylamide-urea gels as described elsewhere (2).

RESULTS

Use of Dimethyl Sulfate as a Probe for DNA-Protein Interactions According to Gilbert, Maxam, and Mirzabekov: Gilbert et al. (4) have shown that the lac repressor, when bound to the lac operator, blocks methylation of specific A and G residues and enhances methylation of certain other A and G residues.

It is possible to pinpoint the methylated purines provided the sequence is known. This is because methylation destabilizes the purine-sugar glycosidic bond such that the methylated purine readily comes off the sugar. The depurinated chain is then cleaved by, for example, treatment with alkalai. Modifications of these procedures permit one to cleave specifically at methylated G residues or, alternatively, predominantly at A residues. In practice, duplex DNA of known sequence terminally labelled at the appropriate end is methylated in the presence of the ligand under controlled conditions in such a way that an average of one purine or less per labelled molecule is affected. After the methylation reaction, the DNA is depurinated and the strands broken at every site where a depurination has occurred. Fractionation of the degraded DNA on a denaturing acrylamide-urea gel yields a reproducible set of DNA bands. Each band on the gel reflects the occurrence and location of a purine in the DNA sequence with reference

to the labelled terminal nucleotide. If some of the purines have been shielded by a DNA-binding ligand at the time of methylation, the bands corresponding to these purines will be either weakened or abolished. If the binding of the ligand results in increased access to some purines at the time of methylation, the bands corresponding to these purines will be enhanced as compared to a control.

Operator-Containing Restriction Fragments Are Prepared by Strategic Use of Restriction Endonucleases: A number of restriction endonucleases cleave λ DNA within and around the operators to generate various restriction fragments bearing whole operator sequences or parts thereof. Figure 1a shows some relevant restriction cleavage sites in and around the two operators. The following restriction fragments were used for analyzing the left operator: Hin 320 (which contains the hypothetical binding site O_L1) and Hin 1125 (O_L2 plus O_L3). For analyzing the right operator, the following restriction fragments were used: Alu 180 (entire O_R), Hin 550 (O_R1), Hin 375 (O_R2 and O_R3), and Hph 75 (most of O_R). These restriction fragments were labelled at both ends with ^{32}P and then cut with various restriction endonucleases to isolate secondary restriction fragments labelled at the appropriate (operator) end. For example, Hin 320 (O_R1) was labelled at both ends and then digested with HaeIII to generate Hae/Hin 140 as shown in Figure 1b. This fragment, which has the Hin end labelled, was isolated by gel electrophoresis and used in a protection experiment.

Repressor Affects the Methylation of G's But Not That of A's within Operators: Figure 2a shows examples of the effects of repressor on methylation of purines in λ operators. For the experiment of 2a, a DNA fragment bearing O_R1 was used. This fragment, Hae/Hin 140, was labelled at the Hin end, and the figure shows the products of cleavage at G residues. The band corresponding to each G in the sequence is identified on the figure as far as resolution permits. Thus, the lowest band in the first vertical column in Figure 2a corresponds to the G at position 16 in the nucleotide sequence (numbered +16 in Figure 3) counting from the labelled end; the band

immediately above that corresponds to the G at position 17 (+17 in Figure 3) and so on. A comparison of the first vertical column in Figure 2a, which represents a control experiment with no repressor with the second vertical column, which represents an experiment in which repressor was added before methylation, reveals that the repressor suppresses or abolishes the bands corresponding to G's at positions 17, 19, 20, and 22. Repressor has blocked the methylation of these G's. In contrast, the methylation of the G at position 16 is enhanced as reflected by increased intensity of the band as compared to the control. Other G's in the sequence are unaffected. Figure 2b represents a similar experiment with a DNA fragment containing part of the left operator in which the methylated DNA was subjected to an A-specific cleavage. This fragment, Hae/Hin 190 includes O_L2 and O_L3, and was labelled at the Hin end. The figure reveals that repressor has no significant effect on the methylation of any A's. Similar results were obtained with various other operator-containing restriction fragments. In all cases, repressor had an effect only on the methylation of G's.

Figure 2c represents the results of an experiment in which a DNA fragment containing the entire right operator (Alu/Hha 160) was subjected to G-specific cleavage after methylation. The figure shows that repressor protects G residues in three different regions within the binding sites O_R1, O_R2, and O_R3, but not in the spacers between those sites (see Figure 3). In addition, Figure 2c suggests the order in which the repressor binds to the three sites in O_R and therefore, the relative affinity of the sites for the repressor. At the repressor concentration used, the shielding of the blockable G's is complete in O_R1, almost complete in O_R2 and partial in O_R3. At sufficiently high repressor concentrations, the shielding of blocked G's in all three sites is complete (not shown). Figure 2c also reveals a puzzling phenomenon. At a certain given concentration repressor completely blocks some G's in some sites (in this example, O_R2) and yet has only a partial effect on other G's in the same site. However, all blockable G's are completely shielded if the repressor concentration is increased.

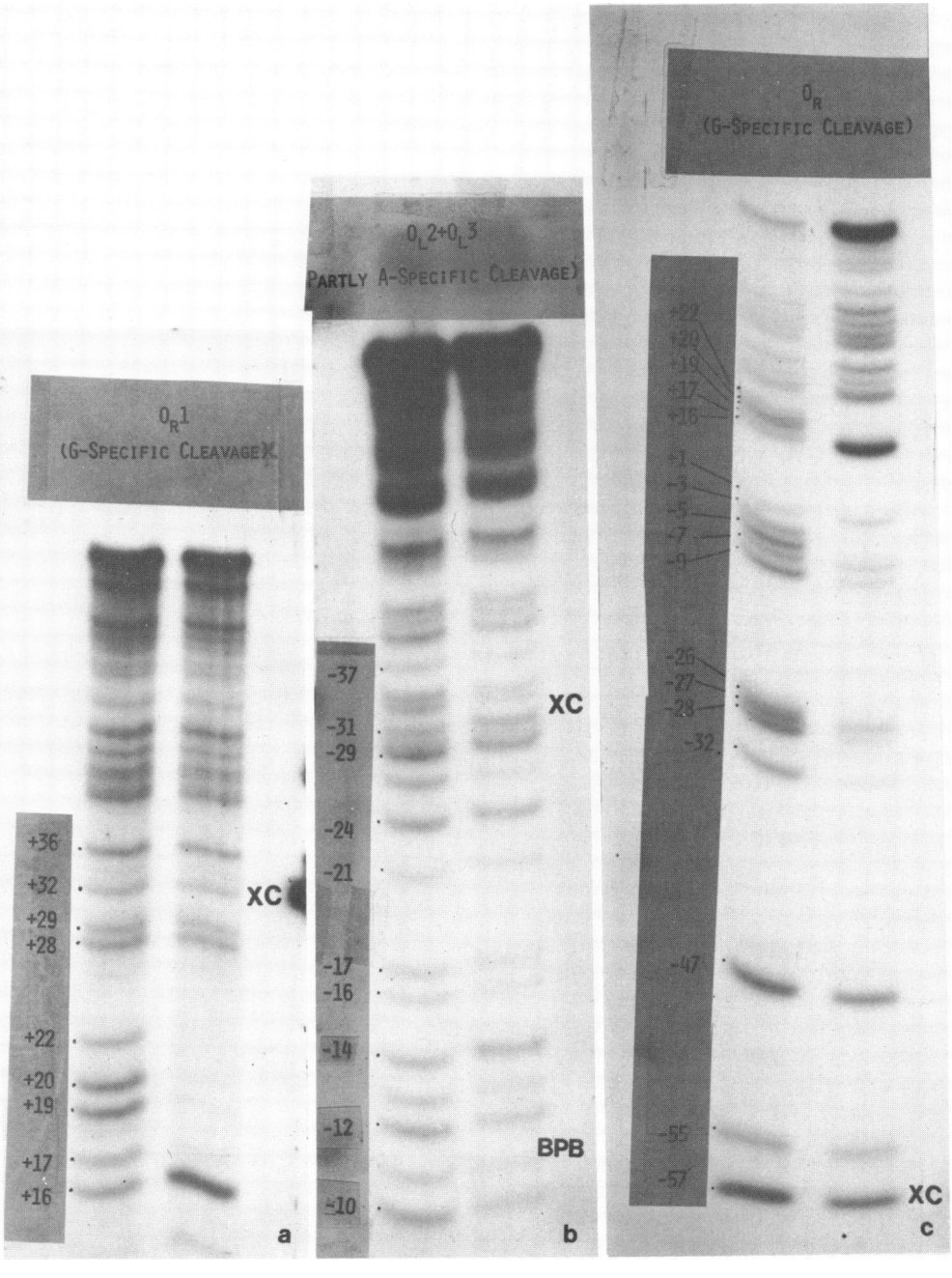
O_R1
(G-Specific Cleavage)
+36
+32
+29
+28
+22
+20
+19
+17
+16
XC
a
O_L2+O_L3
Partly A-Specific Cleavage)
-37
-31
-29
-24
-21
-17
-16
-14
-12
-10
XC
BPB
b
O_R
(G-Specific Cleavage)
+22
+20
+19
+17
+16
+1
-3
-5
-7
-9
-26
-27
-28
-32
-47
-55
-57
XC
c

Figure 2

Gel fractionation of cleavage products from various operator-containing DNA fragments methylated in the absence and presence of λ repressor. Each DNA band is due to a break at a methylated purine. The numbers identify the bands corresponding to the purines in the DNA sequence, as given in Figure 3. XC and BPB are the dye markers xylene cyanol and bromphenol blue respectively. See text for experimental procedures.

a. Effect of repressor on the methylation of G's in Hae/Hin 140 (labelled at Hin end), a DNA fragment containing the primary repressor binding site of the right operator (O_R1). The DNA was methylated in the presence of a 20-fold molar excess of repressor over operator, and then subjected to G-specific cleavage (second vertical column). The first vertical column represents a control experiment from which repressor was omitted.

b. Effect of repressor on the methylation of A's in Hin/Hae 190 (labelled at Hin end), a DNA fragment containing O_L2+O_L3. DNA was methylated in the presence of a 30-fold molar excess of repressor, and then subjected to a partially A-specific cleavage (second vertical column). The first vertical column represents a control experiment without repressor.

c. Effect of repressor on the methylation of G residues in Alu/Hha 160 (labelled at the Alu end), a fragment containing the entire right operator (O_R). After methylation in the presence of a 30-fold molar excess of repressor, the DNA was subjected to a G-specific cleavage (second column). The first column represents a control experiment with no repressor.

Figure 3 shows the entire nucleotide sequence of both λ operators. The startpoints of transcription of genes N, tof, and cI are indicated, and the three presumed repressor binding sites within each operator are boxed. Both strands of the entire O_R sequence have been tested in experiments such as those described in Figure 2. Using the DNA fragments described in Table 1, at O_L, the upper strand in Figure 3 to the right of the Hin cut, and the lower strand to the left of the Hin cut, have been tested. As indicated in Figure 3 and its legend, repressor affects methylation of G residues only, and only of those G residues within the presumed binding sites. The usual effect of repressor is to suppress methylation, but there are two instances of enhancement and four where repressor has no effect.

DISCUSSION

The results presented in this communication show that the repressor affects the methylation of G's in λ operators but has no effect on the methylation of A's. Apparently the repressor

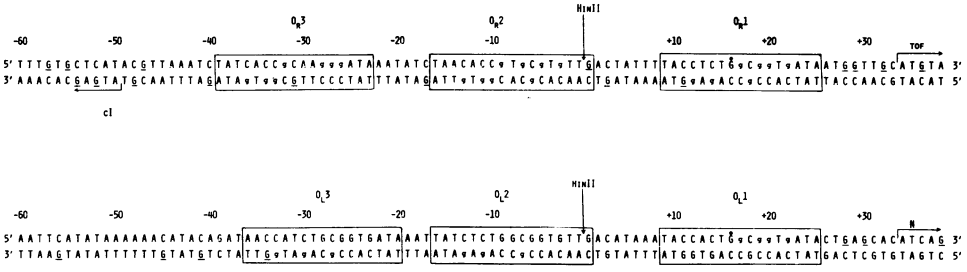

Figure 3

Nucleotide sequence of the phage λ operators. (Taken from reference 2, except for the 8 bp sequence in O_L between -52 and -60, which is from our unpublished results.) Note that the orientation of O_L is reversed from its usual presentation on the λ genome. The proposed 17 bp long repressor-binding sites are boxed. G's whose methylation is suppressed by repressor are indicated by small case lettering. G residues whose methylation is enhanced by the repressor are marked with asterisks. Unaffected G's are underlined. Other G's have not been tested. For convenience, base pairs are numbered from either side of the HinII cutting site in each operator.

penetrates the major groove as it binds to the operator and is in either physical contact with or in close proximity to the affected G residues. The fact that the methylation of A's is not affected may mean that λ repressor does not penetrate the minor groove of DNA.

The distribution of repressor-shielded G's in various restriction fragments bearing operator sequences provides direct evidence that the repressor is in actual contact with the hypothesized binding sites in each operator (Figure 3). All the G's whose methylation is affected in the presence of repressor are within the proposed binding sites, there being no effect on the G's in the spacers or on the G's that occur outside the operators. (It should be noted that each spacer contains no more than one G residue.) The fact that there are no repressor-affected G's beyond the third site in each operator (O_L3 or O_R3) reinforces our recent conclusions that each operator consists of only three repressor-binding sites (1). The extent of shielding of G's at a given repressor concentration is apparently a function of the affinity between a binding site and the repressor. Thus, at repressor concentrations where susceptible G's in the primary binding sites (O_L1 or O_R1) are shielded, the blocking

of susceptible G's in the two secondary sites is only partial (Figure 2c). Complete blocking of G's in the two secondary sites (O_L2+O_L3 or O_R2+O_R3) requires higher repressor concentrations. Thus our present results support the conclusion that O_L1 and O_R1 have higher affinity for repressor than do the remaining sites (7,2). Repressor has a positive effect on the methylation of the G at position +16 in both O_L1 and O_R1. No such effect was observed in other parts of either operator. Gilbert *et al.* (4) have suggested that a DNA binding protein might enhance methylation by causing a local alteration in the structure of double stranded DNA or by forming a hydrophobic pocket that traps the methylating reagent.

An examination of the pattern of shielded G's in the various sites in both operators reveals an interesting regularity. The sequence $5'\begin{matrix}CGGTG\\GCCAC\end{matrix}3'$ is present in all six sites from both operators, although not in the same orientation. All G's in this sequence (as far as tested) are shielded by the repressor. Aligning the sequences of the six sites so as to bring the CGGTG sequences in the same orientation as shown in Figure 4 reveals the following points: each repressor-binding site consists of a half-section that has a more or less conserved nucleotide sequence (i.e., the sequence $\begin{matrix}TATCACCGC\\ATAGTGGCG\end{matrix}$), and another half that is more variable. The points of contact in the conserved half are identical in all the sites tested, and apparently are vital for recognition by the repressor. We point out that most of the operator mutations so far sequenced are confined to the conserved half, and in particular, to the $\begin{matrix}CGGTG\\GCCAC\end{matrix}$ sequence (see Figure 4).

It is pertinent to compare the results presented here with those obtained with the *lac* operator-repressor system (4). The most striking difference is that in the *lac* operator, both G's and A's are affected in the presence of *lac* repressor, indicating that the repressor has points of contact in both the major and the minor DNA grooves. *Lac* repressor has positive as well as negative effects on the methylation of both A's and G's. In *lac* the enhanced purines are near the middle of the operator sequence, and in λ the enhanced G's are also near the middle of the operator sequence. In λ, as in *lac*, there is no obvious

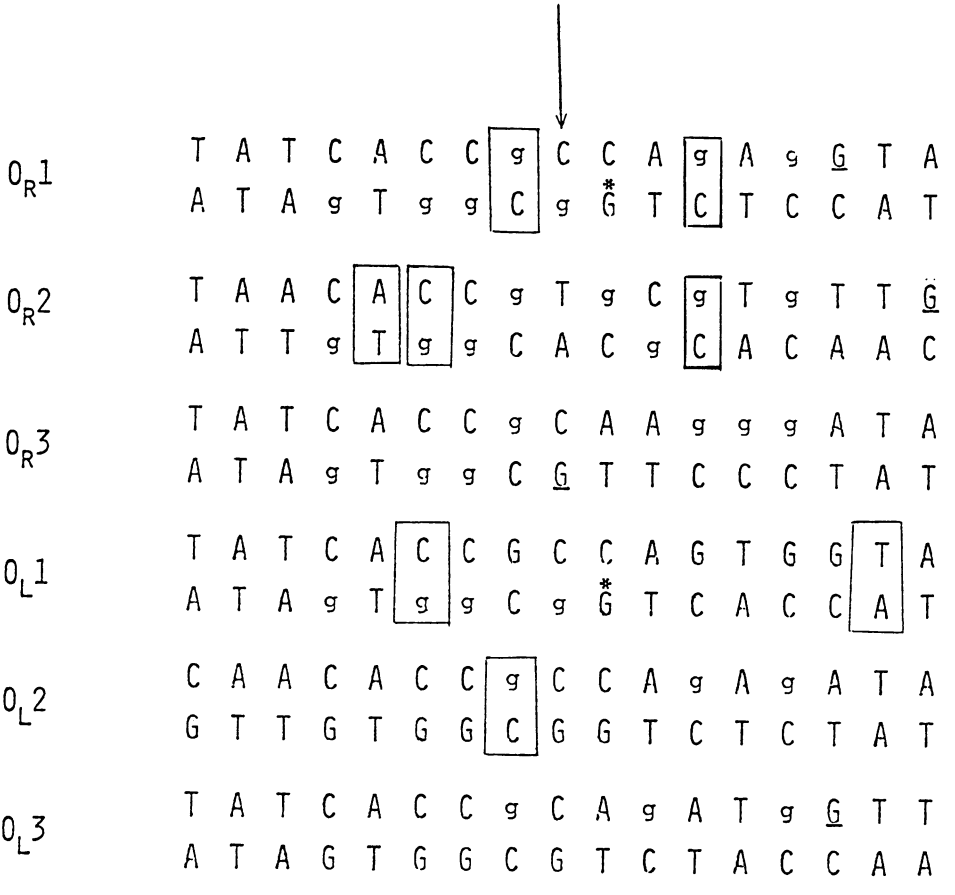

Figure 4
An alignment of the six proposed repressor binding sites from the λ operators so as to bring out similarities in the distribution of those G residues whose methylation is modified by repressor. Protected G's are indicated by small case lettering and enhanced G's are marked with an asterisk. Sites of several known operator mutations are boxed.

symmetric pattern in the distribution of repressor-affected purines within the binding site (see Figure 4), suggesting that the repressor does not fully exploit the symmetrical features of the operator sequence.

REFERENCES

*Dedicated to Jerome Vinograd

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