Phage λ Cro Protein and *cI* Repressor Use Two Different Patterns of Specific Protein-DNA Interactions to Achieve Sequence Specificity *in Vivo*

Nicholas Benson and Philip Youderian

Department of Biological Sciences, University of Southern California, Los Angeles, California 90089-1481

Manuscript received April 25, 1988
Accepted for publication September 14, 1988

ABSTRACT

By assaying the binding of wild-type Cro to a set of 40 mutant λ operators *in vivo*, we have determined that the 14 outermost base pairs of the 17 base pair, consensus λ operator are critical for Cro binding. Cro protein recognizes 4 base pairs in a λ operator half-site in different ways than *cI* repressor. The sequence determinants of Cro binding at these critical positions *in vivo* are nearly perfectly consistent with the model proposed by W. F. ANDERSON, D. H. OHLENDORF, Y. TAKEDA and B. W. MATTHEWS and modified by Y. TAKEDA, A. SARAI and V. M. RIVERA for the specific interactions between Cro and its operator, and explain the relative order of affinities of the six natural λ operators for Cro. Our data call into question the idea that λ repressor and Cro protein recognize the consensus λ operator by nearly identical patterns of specific interactions.

CRO protein, the product of the *cro* gene of coliphage λ, is a small polypeptide 66 amino acid residues long (ROBERTS *et al.* 1977; HSIANG *et al.* 1977; TAKEDA, FOLKMANIS and ECHOLS 1977). Cro monomers assemble into active dimers *in vivo*, that bind six specific DNA sites (operators) on the λ genome with differential affinities. These operators control the expression of the two phage λ early promoters, P_L and P_R [see PTASHNE *et al.* (1980) and GUSSIN *et al.* (1983) for reviews]. The structure of Cro protein has been solved by X-ray crystallographic analysis to 2.8A resolution (ANDERSON *et al.* 1981), and has been refined subsequently to 2.2A (OHLENDORF *et al.* 1982). These studies show that Cro monomer is composed of three strands of antiparallel β-sheet and three α-helices joined by short surface turns, plus a short, disordered carboxyl-terminal arm (Figure 1).

In the Cro dimer, the third α-helices (α_3; Figure 1) in symmetrically related monomers are separated by a center-to-center distance of 34A, the distance between successive major grooves of B-form DNA (ANDERSON *et al.* 1981). The phenotypes of mutations that change amino acid residues in α_3 implicate this secondary substructure of Cro in specific DNA-binding (PAKULA, YOUNG and SAUER 1986). Together, these results suggest that the two subunits of the Cro dimer contact nucleotide base pairs (bp) located in successive, adjacent major grooves on the same face of the DNA duplex. The results of chemical and enzymatic protection and interference experiments also indicate that the functional groups on DNA implicated in binding are nearly symmetrically disposed in two successive major grooves (JOHNSON, MEYER and PTASHNE 1978; JOHNSON, PABO and SAUER 1980).

To model how Cro binds the λ operator, OHLENDORF *et al.* (1982) have proposed that side chains of residues in α_3 make a specific pattern of contacts with operator DNA. This model was constructed initially by using computer graphic approaches to fit the binding surface of Cro defined by the crystal structure with B-form operator DNA, and has been refined successively to account for the results of detailed kinetic and thermodynamic measurements of the binding of wild-type Cro to various operator sequences (TAKEDA *et al.* 1983; KIM *et al.* 1987; SARAI and TAKEDA 1987; TAKEDA, SARAI and RIVERA 1988). A second model, proposed more recently by HOCHSCHILD and PTASHNE (1986) and elaborated by HOCHSCHILD, DOUHAN and PTASHNE (1986), is based on a different set of genetic and chemical modification data, involving the binding of mutant Cro proteins to various operator sequences. This alternative model argues that a subset of the specific contacts Cro makes with the λ operator is identical to a subset of the contacts made by λ *cI* repressor. The two models are summarized in Figure 2.

To assess the predictions of each model *in vivo*, we examine the binding of wild-type Cro *in vivo* to each member of a set of variant λ operators derived from a consensus, 17-bp operator that is symmetric about its central bp, and to which Cro binds with high affinity *in vitro* (KIM *et al.* 1987). Each member of this set of operators differs from the consensus by a pair of symmetric substitutions. Our previous analysis of λ *cI* repressor binding to these operators has shown that bp substitutions at six positions in an operator half-site disrupt *cI* repressor binding, and define these six bp as critical for binding. Given the simple assumption that loss of a pair of specific contacts (hydrogen bonds

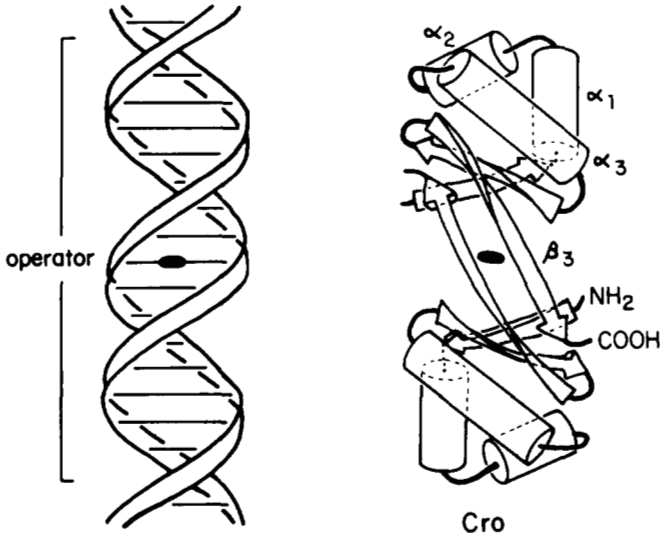

FIGURE 1.—Cro protein recognizes features of successive major grooves of B-form DNA. The schematic drawing, from TAKEDA *et al.* (1983), shows two successive major grooves on B-form DNA and a Cro dimer viewed orthogonally with respect to their dyad axes. Note that the angle of tilt between the α_3 helices and dyad axes of Cro is similar to that of the angle of incidence between the major axis and the screw axes of the major grooves in the λ operator. The side chains of more amino-terminal residues of α_3 should contact positions more distal to the center of dyad symmetry of the operator (see Figure 2).

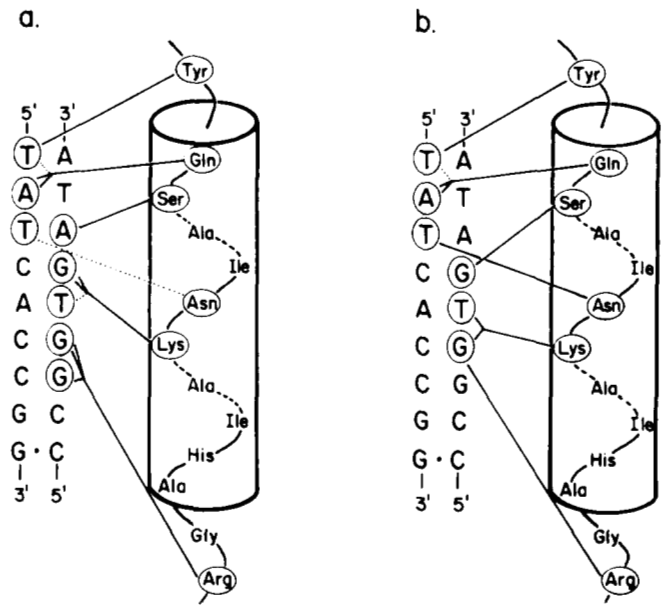

FIGURE 2.—Models proposed for Cro binding to the λ operator. For each model, the α_3 helix of Cro is depicted to the right of the consensus operator half-site sequence; the dyad axis passes through position 9 of the operator, indicated by the dot. Solid lines connecting amino acids of Cro with nucleotide bases indicate hydrogen bonds involved in sequence-specific recognition; dashed lines indicate critical van der Waals contacts. The model of OHLENDORF *et al.* (1983) as modified by TAKEDA, SARAI and RIVERA (1988) is shown in (a); that proposed by HOCHSCHILD and PTASHNE (1986) is shown in (b). The main difference between the two models is that Ser28 is predicted to contact bp 3 of the operator in the model of TAKEDA, SARAI and RIVERA (a), whereas Ser28 contacts bp 4 in the model of HOCHSCHILD and PTASHNE (b). Lys32 and Arg38 are predicted to interact with different bp in the two models.

or van der Waals interactions) between a repressor dimer and the operator is sufficient to result in a loss of binding by our assay, our results *in vivo* corroborate the detailed predictions made by LEWIS *et al.* (1983) of how repressor might interact with the operator (BENSON, SUGIONO and YOUDERIAN 1988).

Given the same assumption, the results of our analysis of Cro binding *in vivo* are nearly perfectly consistent with the predictions of the model proposed for Cro binding by OHLENDORF *et al.* (1982) as revised by TAKEDA, SARAI and RIVERA (1988). We argue that the data obtained by HOCHSCHILD and PTASHNE (1986) can be reinterpreted, and suggest that Cro may use its surface amino acid side chains to make different sets of specific contacts with operator half-sites that differ in sequence.

MATERIALS AND METHODS

Bacteria, phage and plasmids: Bacterial strains, derivatives of *Salmonella typhimurium* LT2 or *Escherichia coli* K12, and challenge phages have been described previously (BENSON, SUGIONO and YOUDERIAN 1988), with the exception of Salmonella strain MS1868/F′*lacI*^{Q1} carrying the plasmid source of Cro protein, pAP101 (PAKULA, YOUNG and SAUER 1986). Plasmid pAP101 carries an operon fusion of the *lacUV5* promoter to the *cro* gene. Media, enzymes, chemicals, general phage techniques, and challenge phage constructions were as described previously (BENSON, SUGIONO and YOUDERIAN 1988).

Challenge phage infections: Challenge phage infections were performed as described (BENSON, SUGIONO and YOUDERIAN 1988), with several modifications. MS1868/F′*lacI*^{Q1} (pAP101) was grown in LB medium supplemented with 50 μg/ml ampicillin at 37° to a density of 2×10^8/ml, β-isopropylthiogalactoside (IPTG) was added to a final concentration of 10^{-5} M, and the cells were grown to a density of 4×10^8/ml. Challenge phage were added to give an input multiplicity of infection of 20 phage/cell, and allowed to adsorb for 20 min at 25°. Appropriate dilutions of infected cells were spread on green indicator plates with ampicillin (50 μg/ml), kanamycin sulfate (20 μg/ml), and with or without 10^{-5} M IPTG. Plates were scored after incubation for 20 hr at 37°. The frequency of lysogeny of cells infected with challenge phage is the concentration of surviving cells divided by the concentration of infected cells. All frequencies of lysogeny are average values determined from at least three different experiments, and were found to vary by less than a factor of five from experiment to experiment.

Unlike the case for λ *cI* repressor-producing cells, the frequency of lysogeny of Cro-producing cells infected with challenge phages shows an extremely steep dependence on IPTG concentration [see BENSON, SUGIONO and YOUDERIAN (1988) for comparison]. In the presence of IPTG at concentrations below 10^{-5} M, few ($<10^{-5}$) pAP101-carrying host cells survive challenge phage infection; at IPTG concentrations higher than 10^{-5} M, the overproduction of Cro protein or mRNA inhibits cell growth (data not shown).

RESULTS

Order of affinities of the natural λ operators for Cro protein: Challenge phages are derivatives of P22

TABLE 1

Frequencies of lysogeny of Cro-producing cells infected with challenge phages carrying the natural λ operators

Operator	Sequence	IPTG 10 μM	IPTG 0 μM
ref	TATCACCGGCGGTGATA	0.1	4×10^{-6}
O_{L1}	TACCACTGGCGGTGATA	4×10^{-5}	$<10^{-7}$
O_{L2}	TATCTCTGGCGGTGTTG	$<10^{-7}$	$<10^{-7}$
O_{L3}	AACCATCTGCGGTGATA	$<10^{-7}$	$<10^{-7}$
O_{R1}	TACCTCTGGCGGTGATA	$<10^{-7}$	$<10^{-7}$
O_{R2}	TAACACCGTGCGTGTTG	$<10^{-7}$	$<10^{-7}$
O_{R3}	TATCACCGCAAGGGATA	0.3	3×10^{-6}

Cells producing Cro protein were infected with challenge phages carrying the listed operator sequences. Numbers in the columns are frequencies of lysogeny of Cro-producing cells (the number of surviving cells divided by the number of infected cells) from challenge phage infections in the absence or presence (10^{-5} M) of IPTG, performed as described in MATERIALS AND METHODS.

TABLE 2

Frequencies of lysogeny of Cro-producing cells infected with challenge phages carrying symmetric λ operators

Operator	Sequence	IPTG 10 μM	IPTG 0 μM
ref (9G)	TATCACCGGCGGTGATA	0.1	4×10^{-6}
18 bp	TATCACCGGCCGGTGATA	$<10^{-7}$	$<10^{-7}$
16 bp	TATCACCG..CGGTGATA	$<10^{-7}$	$<10^{-7}$
9C	TATCACCGCCGGTGATA	0.2	6×10^{-6}
9A	TATCACCGACGGTGATA	0.2	5×10^{-6}
9T	TATCACCGTCGGTGATA	0.2	6×10^{-6}

Cro-producing cells were infected with challenge phages carrying one of the synthetic λ operators with the sequences shown. Numbers are frequencies of lysogeny, as in Table 1. ref = reference-type operator.

Kn9 arc-amH1605 phage carrying a substitution of an alternative DNA-binding site for the P22 *mnt* operator. Upon infection with a challenge phage at high multiplicity, a Salmonella host will survive if and only if it produces a protein that can bind the substituted site. The fraction of cells that survive challenge phage infection is a measure of how well the substituted site is occupied by a specific DNA-binding protein (BENSON *et al.* 1986; BASS *et al.* 1987; BENSON, SUGIONO and YOUDERIAN 1988; LEBRETON *et al.* 1988).

We have shown that challenge phages with the consensus λ operator efficiently lysogenize a host that produces λ *cI* repressor from a plasmid (BENSON *et al.* 1986; BENSON, SUGIONO and YOUDERIAN 1988). We tested whether challenge phages carrying the consensus λ operator or each of the six natural operators could lysogenize a similar host that produces Cro protein. Table 1 shows that, among these seven operators, the consensus and O_{R3} operators have the highest affinity for Cro protein. The O_{L1} operator, bound best by λ repressor among this set of operators, is bound poorly but detectably by Cro. We cannot detect the binding of Cro to any of the four remaining natural operators, O_{L2}, O_{L3}, O_{R1}, and O_{R2}, and cannot order their relative affinities by this binding assay *in vivo*. The order of affinities of these operators for Cro (consensus, $O_{R3} > O_{L1} > O_{R1}$, O_{L2}, O_{L3}, O_{R2}) is in agreement with that determined *in vitro* by KIM *et al.* (1987), with the exception that O_{R1} was reported to be better than O_{L1}. Reexamination of the primary data obtained *in vitro* has shown that the relative affinity of O_{L1} is slightly better than that of O_{R1}, and the orders of affinity determined *in vivo* are perfectly consistent with those determined *in vitro* (Y. TAKEDA, personal communication).

Cro protein, like *cI* repressor, prefers to bind an asymmetric operator: The 17-bp consensus operator bound by λ repressor and Cro protein has an asymmetric, central G:C bp (Table 1). An axis of symmetry through this bp relates the 16 bp in the two operator half-sites perfectly. Symmetrically related subunits of a Cro dimer are thought to recognize the two operator half-sites nearly identically (JOHNSON, MEYER and PTASHNE 1978; JOHNSON, PABO and SAUER 1980; ANDERSON *et al.* 1981). To determine whether Cro protein, like Lac repressor, prefers to bind a symmetric site (SADLER, SASMOR and BETZ 1983), we made two perfectly symmetric variants of the consensus operator 16 and 18 bp in length. As shown in Table 2, neither the shorter nor the longer operators bind Cro so well as the 17 bp reference-type operator. From this result we conclude that Cro prefers to bind an asymmetric, 17-bp operator, like λ *cI* repressor (BENSON, SUGIONO and YOUDERIAN 1988). Cro cannot "adjust itself" to recognize pairs of operator half-sites when these half-sites are incorrectly spaced along the DNA helix, a possibility that has been suggested by OHLENDORF *et al.* (1982). Thus, the relative spacing and rotational disposition of the λ operator half-sites with respect to one another on the surface of B-form DNA are critical for Cro binding.

How critical is the central, asymmetric base pair for Cro binding? Table 2 shows that changing the central, asymmetric bp has no discernible effect on Cro binding, consistent with other results that indicate Cro does not interact with functional groups of the central three bp (positions 8 and 9) of the operator (JOHNSON, MEYER and PTASHNE 1978; JOHNSON, PABO and SAUER 1980).

Operator determinants of Cro binding: Presumably each half-site of a λ operator is recognized in a similar way by a symmetrically related monomer of a Cro dimer. Operators differing from the consensus by two symmetric mutations should present similar DNA surfaces in each operator half-site to each Cro monomer in a bound dimer. Therefore, we measured the ability of Cro to bind each of a set of variant λ

TABLE 3

Frequencies of lysogeny of Cro-producing cells infected with challenge phages carrying symmetric, variant λ operators

Operator	Sequence	IPTG 10 μM	IPTG 0 μM
ref	TATCACCGGCGGTGATA	0.1	4×10^{-6}
1G*	GATCACCGGCGGTGATC	$<10^{-7}$	$<10^{-7}$
1A	AATCACCGGCGGTGATT	0.2	2×10^{-6}
1C	CATCACCGGCGGTGATG	4×10^{-7}	$<10^{-7}$
2G	TGTCACCGGCGGTGACA	$<10^{-7}$	$<10^{-7}$
2T	TTTCACCGGCGGTGAAA	$<10^{-7}$	$<10^{-7}$
2C	TCTCACCGGCGGTGAGA	$<10^{-7}$	$<10^{-7}$
3G	TAGCACCGGCGGTGCTA	$<10^{-7}$	$<10^{-7}$
3A	TAACACCGGCGGTGTTA	0.2	2×10^{-6}
3C	TACCACCGGCGGTGGTA	$<10^{-7}$	$<10^{-7}$
4G	TATGACCGGCGGTCATA	$<10^{-7}$	$<10^{-7}$
4A	TATAACCGGCGGTTATA	$<10^{-7}$	$<10^{-7}$
4T	TATTACCGGCGGTAATA	$<10^{-7}$	$<10^{-7}$
5G	TATCGCCGGCGGCGATA	$<10^{-7}$	$<10^{-7}$
5T	TATCTCCGGCGGAGATA	5×10^{-7}	$<10^{-7}$
5C	TATCCCCGGCGGGGATA	$<10^{-7}$	$<10^{-7}$
6G	TATCAGCGGCGCTGATA	$<10^{-7}$	$<10^{-7}$
6A	TATCAACGGCGTTGATA	$<10^{-7}$	$<10^{-7}$
6T	TATCATCGGCGATGATA	$<10^{-7}$	$<10^{-7}$
7G	TATCACGGGCCGTGATA	$<10^{-7}$	$<10^{-7}$
7A	TATCACAGGCTGTGATA	$<10^{-7}$	$<10^{-7}$
7T	TATCACTGGCAGTGATA	0.04	4×10^{-6}
8A	TATCACCAGTGGTGATA	0.3	9×10^{-6}
8T	TATCACCTGAGGTGATA	0.3	8×10^{-6}
8C	TATCACCCGGGGTGATA	0.2	6×10^{-6}

Cro-producing cells were infected with challenge phages carrying one of the synthetic λ operators with the sequences shown. Numbers are frequencies of lysogeny, as in Table 1. This analysis was complicated by the fact that one of the substitutions (1, T:A→G:C; operator 1G, indicated by the asterisk) results in the modification of three adjacent base pairs, since it creates a site recognized by Salmonella Dam methylase (5′ GATC 3′), in which adenine C6-amino groups are methylated (GOMEZ-EICHELMANN 1979). In a *dam*$^+$ host, this change not only replaces the T:A base pair at position 1 with G:C, but also results in the methylation of the C6 amino groups of adenine in base pairs 2 and 3. When methylated, this operator sequence does not bind either Cro protein or λ repressor. As yet, we have been unable to assay binding in a *dam*$^-$ Salmonella host. ref = reference-type (consensus) operator.

operators that differ from the consensus operator by pairs of single bp changes (Table 3). As summarized in Figure 3, 7 bp in the λ operator (1 through 7) are critical for Cro binding, since challenge phages carrying operators with changes in these bp cannot lysogenize a Cro-producing host. Four of these bp (2, 4, 5, and 6) are particularly critical for binding, because all three substitutions at each of these operator positions abolish binding.

What the challenge phage assay tells us in the case of Cro is simply whether operator changes have severe effects on binding or not (*i.e.*, reduce the affinity of the interaction substantially *in vivo*). We make no

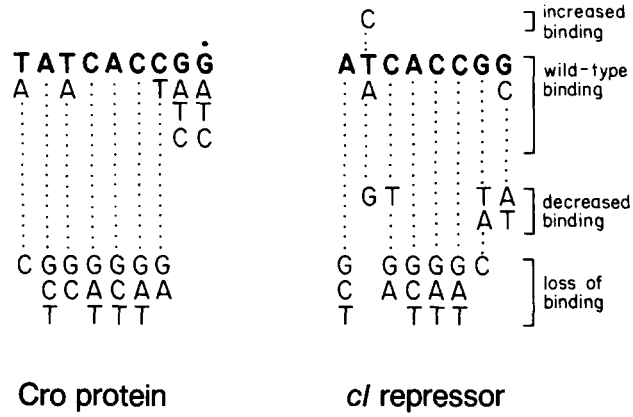

FIGURE 3.—Symmetric base pair changes at six positions in the λ operator impair Cro binding. Cells producing Cro were infected with challenge phages differing from the reference-type operator by symmetric pairs of base pair substitutions. The results of these infections are represented in two dimensions. The sequence of the top strand of the left, reference-type half-site is represented by bold letters; the dyad axis passes through position 9 of the operator, indicated by the dot. Immediately below the reference sequence are listed changes that do not change operator strength (negligible). Below the reference sequence are listed the changes that reduce the ability of Cro to bind to more than 10^4-fold (severe). The data for *cI* repressor (BENSON, SUGIONO and YOUDERIAN 1988) are shown for comparison; some operator changes fall into a third, intermediate category in terms of their ability to be bound by *cI* repressor, changes that reduce survival less than 10^4-fold (mild). The 3C change is shown above the consensus half-site for *cI* repressor, since it results in an operator with higher affinity for repressor.

claims that the numbers we obtain as an indication of relative affinities measured by a genetic assay *in vivo* have anything to do with relative affinities measured *in vitro*. In fact, this is something we do not wish to do, particularly in view of the results of NELSON and SAUER (1985) showing that affinities measured for λ repressor mutants *in vitro* may have a limited relation to the physiologically important situation *in vivo*. However, we note that the simplest interpretation of our results is consistent with the detailed model evolved for Cro binding from the systematic analysis of Cro binding to operators differing from the natural O_{R1} operator by single base pair substitution mutations *in vitro* (TAKEDA, SARAI and RIVERA 1988).

Cro binding to asymmetric operators: Each of the two natural operators bound by Cro protein, O_{R3} and O_{L1}, are composed of two half-sites, one of which has the consensus sequence, and one of which differs from the consensus by multiple, asymmetric mutations. To probe how individual substitutions in the nonconsensus, or variant, half-sites of these operators affect Cro binding, we synthesized four asymmetric operators composed of one consensus half-site and one variant half-site differing from consensus by a single substitution found in the variant half-sites of O_{R3} or O_{L1}. Table 4 shows that operators carrying the asymmetric changes 3C or 5T are bound by Cro almost as well as the consensus operator, whereas the asymmetric 5C

TABLE 4

Frequencies of lysogeny of Cro-producing cells infected with challenge phages carrying asymmetric λ operators

Operator	Sequence	IPTG 10 μM	IPTG 0 μM
ref	TATCACCGGCGGTGATA	0.1	4×10^{-6}
3C (asym)	TACCACCGGCGGTGATA	0.02	5×10^{-7}
5C (asym)	TATCCCCGGCGGTGATA	0.06*	$<10^{-7}$
5T (asym)	TATCTCCGGCGGTGATA	0.06	2×10^{-6}
7T (asym)	TATCACTGGCGGTGATA	0.08	4×10^{-6}

Cro-producing cells were infected with challenge phages carrying either the consensus, reference-type operator (ref), or one of the asymmetric (asym) operator sequences shown. Numbers are frequencies of lysogeny, as in Table 1. An asterisk indicates that colonies appear after 40 hr of incubation. Since these survivors are unstable lysogens carrying the challenge phage as prophage (and form dark green colonies on green indicator plates), we interpret their slow growth to mean that the amount of repressor produced under these conditions is near the threshold required to saturate a particular operator (see BENSON, SUGIONO and YOUDERIAN 1988).

operator is bound more poorly than consensus. When each of these mutations is present as a pair of symmetric substitutions, it abolishes detectable binding (Table 3). The asymmetric 7T change does not appear to affect Cro binding significantly, consistent with the finding that the symmetric mutant 7T operator binds Cro well (Table 3).

The mutant 5C (asymmetric) operator binds Cro, whereas the 5C (symmetric) operator does not. Survivors resulting from the challenge of our Cro-producing host with a phage carrying the 5C (asymmetric) operator form unstable lysogens, as is the case for other repressor-producing hosts that produce just enough protein to barely saturate a site (BENSON, SUGIONO and YOUDERIAN 1988; HUGHES, YOUDERIAN and SIMON 1988). The 5C (asymmetric) operator has just sufficient affinity to be bound by Cro under our conditions *in vivo*.

DISCUSSION

To define the operator determinants of Cro binding *in vivo*, we have tested the ability of Cro to bind each operator among a set of λ operators differing from consensus by symmetric pairs of bp substitutions. At least one symmetric pair of substitutions at each of seven operator positions prevents Cro binding, indicating that these seven bp in an operator half-site are critical for the formation or stability of the Cro/operator complex (Table 3). Unlike the case for λ *cI* repressor (BENSON, SUGIONO and YOUDERIAN 1988), none of the symmetric, variant operators derived from consensus binds Cro measurably better than consensus.

When the operator determinants for Cro binding are compared with those for λ *cI* repressor binding (Figure 3), one can see that at each operator position, particular substitutions may affect repressor binding, Cro binding, or both. At positions 1, 3, 4, and 7 of the operator, we find that particular substitutions affect *cI* repressor binding differently than Cro binding. Therefore, the two repressors most likely recognize these bp in different ways.

Order of affinity of the natural λ operators for Cro: The orders of affinity for the binding of *cI* repressor and Cro to the six natural operators differ (JOHNSON, MEYER and PTASHNE 1978; JOHNSON, PABO and SAUER 1980). *cI* repressor binds O_{L1} more tightly than O_{R1}, and O_{R1} more tightly than the other four natural sites (BENSON, SUGIONO and YOUDERIAN 1988). In contrast, Cro prefers O_{R3} to O_{L1}, and O_{L1} to other operators (Table 1). Both O_{L1} and O_{R3} are closely related to the consensus site, each differing from consensus by one asymmetric change that, when present as a symmetric pair of mutations, prevents Cro binding (3C and 5C, respectively). Each of the four other natural operators is not bound detectably by Cro, and differs from consensus by two changes that impair Cro binding severely.

Immediate context and Cro binding: The natural λ operators bound best by Cro (O_{R3} and O_{L1}) differ from consensus by multiple, asymmetric mutations clustered in one of their two half-sites. O_{R3} differs from consensus by four mutations, 5C, 7T, 8T, and 9G. Three of these mutations, 7T, 8T, and 9G, have little or no effect on Cro binding when present as symmetric mutations, whereas the fourth, 5C, is detrimental to binding when present as either a symmetric (Table 3) or asymmetric (Table 4) change in the immediate context of a consensus operator half-site. In contrast, in the immediate context of the variant half-site of O_{R3}, the 5C mutation appears to have little or no effect on binding. The O_{L1} operator, bound poorly by Cro (Table 1), differs from consensus by two changes in one of its half-sites, 3C and 7T. Whereas a single, asymmetric 3C change has little effect on Cro binding, when paired with a second change in the same half-site, 7T, that also has little effect on Cro binding, the double mutant operator binds Cro only poorly. In the immediate context of the nonconsensus O_{L1} half-site, the asymmetric 3C change has a more severe effect on binding than when present in a consensus context. It is clear that operator mutations may have different effects on the binding of Cro when found in different immediate contexts, as is the case for the binding of *cI* repressor (BENSON, SUGIONO and YOUDERIAN 1988).

Models for Cro binding: Given the assumption that loss of a pair of specific contacts between a repressor dimer and a pair of operator half-sites is sufficient to result in a loss of binding in our assay, our results *in vivo* are nearly perfectly consistent with the model most recently proposed for Cro binding by OHLEN-

DORF *et al.* (1982), as modified by TAKEDA, SARAI and RIVERA (1988). This model predicts that the side chains of five amino acid residues of Cro, Tyr26, Gln27, Asn31, Lys32, and Arg38, are responsible for the observed specificity of Cro binding.

As originally proposed by OHLENDORF *et al.* (1982), the hydroxyl group of Tyr26 is predicted to donate a proton to form a hydrogen bond with the thymine O4 group at position 1 of the operator, and the side chain of Gln27 may participate in an additional hydrophobic interaction with the methyl group of thymine (TAKEDA, SARAI and RIVERA 1988). We find that Cro can bind the mutant 1A operator, suggesting that Tyr26 may make an alternate set of contacts with a variant A:T bp at position 3. Perhaps when Cro binds the 1A operator, the hydroxyl group of Tyr26 can participate in a pair of hydrogen bonds with the N6-amino and N7-purine groups of adenine, or the N6-amino group of adenine and the O4 group of thymine. The former possibility seems more attractive, since Cro does not bind the 1C operator so well.

Gln27 should both donate a proton to the N7 group and accept a proton from the N6 group of adenine +2, to make two hydrogen bonds with the operator. The model of TAKEDA, SARAI and RIVERA (1988) predicts that all three single bp changes from consensus at position 2 would impair binding, the result we observe.

At position 3 of the operator, Ser28 is thought to make two hydrogen bonds with the N7 and N6 groups of adenine −3. Although this predicts that all three changes from the consensus (3T) at this position might interfere with binding, we find that the symmetric, mutant 3A operator is bound well by Cro. TAKEDA, SARAI and RIVERA (1988) have proposed that Ser28 may make an alternative set of contacts with the 3A operator, hydrogen bonding with the O4 group of thymine −3 and the N6 group of adenine +3. The specificity of recognition at this position may be determined in part by additional hydrophobic contacts between the methyl group of thymine and the side chains of Asn31 and perhaps Lys32. Like operator 3A, operator 3C should present a somewhat congruent pair of donor and acceptor groups to Cro at this position, but is not bound well by Cro. Y. TAKEDA, A. SARAI and V. M. RIVERA (unpublished results) have shown that a mutant Cro with the change Ser28 → Gly binds operators with changes at bp3 with nearly equal affinities *in vitro*, indicating that these repressor and operator mutations are mutually epistatic, and that Ser28 specifically contacts bp 3 of the operator.

In the model of TAKEDA, SARAI and RIVERA (1988), Lys32 may make at least three specific interactions with the operator, its ϵ-amino group participating in two hydrogen bonds with the N7 and O6 groups of guanine at −4, and its hydrophobic side chain making a specific van der Waals contact with the methyl group of thymine −5. These predictions are consistent with our observation that any of the symmetric substitutions at each of these two positions prevents binding. In the alternative model proposed by HOCHSCHILD and PTASHNE (1986), Lys32 is predicted to interact with positions 5 and 6 of the operator different from the observed recognition specificity. (For example, if Lys32 is making a single hydrogen bond with bp 5 (A:T) of the operator, then we might expect that at least one of the changes 5G or 5C should have little effect on Cro binding.)

Arg38 is postulated to form two hydrogen bonds with the N7 and O6 groups of guanine −6, accounting for the specificity observed at this position. In addition, Arg38 may donate a proton to the N7 group of guanine −7 to form a third, specific hydrogen bond with the operator. This hypothesis is consistent with our observation that both the consensus (7C) and variant 7T operators are bound well by Cro, but the 7G and 7A operators are not, implicating bp 7 as a critical determinant of Cro binding.

λ *cI* and Cro proteins do not recognize the consensus λ operator in nearly identical ways. How, then, are we to explain the results of HOCHSCHILD and PTASHNE (1986), that argue *cI* repressor and Cro proteins make nearly identical patterns of interactions with the consensus λ operator? The major differences between the models proposed by HOCHSCHILD and PTASHNE (1986) and that of OHLENDORF *et al.* (1982) is that HOCHSCHILD and PTASHNE argue that Ser28 of Cro contacts position −4 of the operator and, consequently, Lys32 contacts positions 5 and 6. Their argument that Ser28 of Cro makes specific contacts with guanine −4 rests on the results of experiments that examined the binding of Cro to operators differing by three or more substitutions from consensus.

First, HOCHSCHILD and PTASHNE (1986) have found that methylation of the N7 group of guanine −4 in the variant half-site of O_{R1} interferes with the binding of wild-type Cro, but not with the binding of a mutant Ser28 → Ala Cro. This variant half-site differs by three substitutions from consensus (3C, 5T, and 7T), two of which (3C and 5T) are detrimental to Cro binding, immediately flank position 4 of the operator, and alter the immediate context of bp 4 in a most drastic way. OHLENDORF *et al.* (1982) have suggested that particular amino acid side chains of Cro might interact with several alternative bp in different immediate contexts; a logical consequence of this idea is that Cro may recognize consensus and variant half-sites with different networks of specific contacts. Thus, when a Cro dimer binds O_{R1} (albeit poorly), one Cro monomer may make one ("Cro-like") pattern of specific contacts with the more affine, consensus right half-site, whereas the other monomer may be

constrained to make a different ("*cI*-like") pattern of contacts with the less affine, variant left half-site.

The ability of a repressor to make different sets of specific interactions with different operator half-sites is not without precedent. The binding of Lac repressor tetramers to the asymmetric, natural *lac* operator is fundamentally asymmetric; at the central operator positions 7, 8, and 10, which specify critical determinants of Lac repressor binding, different specific contacts are made with the left and right operator half-sites. Thus, symmetric mutations or chemical modifications of the *lac* operator may have *opposite* effects on Lac repressor binding [see SADLER, SASMOR and BETZ (1983) for review].

For both the cases of the Lac repressor/operator and Cro/O_{R1} interactions, we contend that the binding of a repressor to mutant half-sites that differ from a preferred half-site by three or more single bp changes can force the repressor to adopt a pattern of specific weak bonds with the mutant half-site different than the pattern with its preferred half-site. From our perturbation analysis of Cro binding to operators differing from the consensus λ operator by symmetric changes, our data are consistent with the idea that single bp changes in an operator half-site need only result in a simple loss of contacts. The argument that *multiply* mutant half sites may be recognized by alternative patterns of weak bonds is not inconsistent with this interpretation. It is not surprising that when the potential for forming specific, weak chemical bonds is impaired at three or more positions in an operator half-site, the binding surface of a repressor monomer might accede to a more energetically favorable fit to the operator half-site than that dictated merely by the loss of multiple, specific contacts. Moreover, operator substitutions not only may result in the loss of specific contacts, but also may introduce unfavorable potential interactions or alter the local secondary structure of the operator in ways that have more global effects upon binding. Therefore, it is not surprising that Cro protein can make different sets of weak bonds with different operator half-sites, particularly with the poorer half-site of an operator (O_{R1}) to which Cro binding is normally physiologically unimportant.

Second, HOCHSCHILD and PTASHNE (1986) examined the binding of wild-type and mutant Ser28 → Ala Cro proteins to operator O_{R2} and mutant derivatives of O_{R2} *in vivo*. In these experiments, they measured the relative levels of expression of a set of P_r/*lacZ* operon fusions regulated by O_{R2} carried on single-copy λ prophages in the presence and absence of wild-type and mutant Cro. They showed that substitutions at position 4 of O_{R2} are detrimental to the binding of wild-type but not mutant Cro, and concluded from the failure of the mutant Ala28 Cro to discriminate between operators mutant at position 4 that the Ser28 side chain of Cro contacts this position of O_{R2}. However, their experiments are complicated by the fact that the adjacent natural operator, O_{R1}, is also present in each of their operon fusions. To circumvent this problem, they "inactivated" O_{R1} with a single asymmetric 3C change, a change that may not lower the affinity of O_{R1} for Cro significantly below that of O_{R2}, since O_{R1} binds wild-type Cro with at least sevenfold higher affinity than O_{R2} *in vitro* (KIM *et al.* 1987).

Even if we assume that their mutant O_{R1} + 3C operator has an affinity for wild-type Cro somewhat less than that of O_{R2}, we might also expect the results they observe if the model proposed by OHLENDORF *et al.* (1982) is correct, and Ser28 interacts specifically with bp 3. Changes at position 4 of O_{R2} should interfere with repression by wild-type Cro, since these changes disrupt critical contacts between Lys32 and the operator. Furthermore, the model of OHLENDORF *et al.* (1982) would predict that the change in Cro from Ser28 → Ala may be epistatic to the 3C change in O_{R1}. That is, if Ser28 interacts specifically with position 3, both the 3C change and the Ser28 → Ala change should result in the loss of the same specific contacts from the wild-type Cro/operator interaction, and the effects of the Ser28 → Ala change in Cro may mask the effects of the 3C operator mutation. Thus, mutant Cro may bind the O_{R1} + 3C operator with higher affinity than O_{R2}, and consequently fail to discriminate between changes at position 4 of O_{R2}, because these changes have little effect on repression of the P_R/*lacZ* operon fusions, which may be regulated by Cro binding to O_{R1}. This alternative interpretation points out that the results of HOCHSCHILD and PTASHNE (1986) are complicated by the presence of more than one operator on their templates. We have avoided this problem explicitly by examining the binding of Cro to otherwise isogenic templates carrying single λ operators. More important, given that the effects on Cro binding of changes from consensus are dependent upon their immediate contexts, it is not clear that the weaker interactions occurring between Cro and the less affine half-sites of O_{R1}, O_{R2}, or mutant O_{R2} operators are representative of the stronger interactions between Cro and the consensus operator.

The *prima facie* interpretation of our results is that Cro does not recognize its most affine (consensus) operator in a way nearly identical to that of λ *cI* repressor. Although the amino acid residues of both *cI* and Cro implicated in DNA binding are organized into similar ("helix-turn-helix") secondary structures, side chains on the surfaces of the "recognition" (α_3) helices of these two DNA-binding proteins likely are used in different ways to recognize the consensus λ operator. Nonetheless, it is remarkable how both Cro

protein and *cI* repressor use a conserved secondary structure (two α-helices joined by a surface turn) to interact with the λ operator in quite similar manners.

We thank CURTIS ADAMS for expert technical assistance, M. M. SUSSKIND and Y. TAKEDA for their helpful criticisms of the manuscript, and B. W. MATTHEWS for his encouragement. This work was supported by National Institutes of Health grant GM34150 to P.Y.

LITERATURE CITED

ANDERSON, W. F., D. H. OHLENDORF, Y. TAKEDA and B. W. MATTHEWS, 1981 A phage repressor-operator complex at 2.8 A resolution. Nature **316:** 596–601.

BASS, S., P. SUGIONO, D. N. ARVIDSON, R. P. GUNSALUS and P. YOUDERIAN, 1987 DNA specificity determinants of *Escherichia coli* tryptophan repressor binding. Genes Dev. **1:** 565–572.

BENSON, N., P. SUGIONO and P. YOUDERIAN, 1988 DNA sequence determinants of λ repressor binding *in vivo*. Genetics **118:** 21–29.

BENSON, N., P. SUGIONO, S. BASS, L. V. MENDELMAN and P. YOUDERIAN, 1986 General selection for specific DNA-binding activities. Genetics **114:** 1–14.

GOMEZ-EICHELMANN, M. C., 1979 Deoxyribonucleic acid adenine and cytosine methylation in *Salmonella typhimurium* and *Salmonella typhi*. J. Bacteriol. **140:** 574–579.

GUSSIN, G. N., A. D. JOHNSON, C. O. PABO and R. T. SAUER, 1983 Repressor and Cro proteins: structure, function, and role in lysogenization. pp. 93–123. In *Lambda II*, Edited by R. W. HENDRIX, J. W. ROBERTS, F. W. STAHL and R. WEISBERG. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

HOCHSCHILD, A., and M. PTASHNE, 1986 Homologous interactions of λ repressor and λ Cro with the λ operator. Cell **44:** 925–933.

HOCHSCHILD, A., J. DOUHAN III and M. PTASHNE, 1986 How λ repressor and λ Cro distinguish between O_{R1} and O_{R3}. Cell **47:** 807–816.

HSIANG, M. W., R. D. COLE, Y. TAKEDA and H. ECHOLS, 1977 Amino acid sequence of Cro regulatory protein of bacteriophage lambda. Nature **270:** 275–277.

HUGHES, K. T., P. YOUDERIAN, and M. I. SIMON, 1988 Phase variation in Salmonella: analysis of Hin recombinase and *hix* recombination site interactions *in vivo*. Genes Dev. **2:** 937–948.

JOHNSON, A. D., B. J. MEYER and M. PTASHNE, 1978 Mechanism of activation of the cro protein of bacteriophage λ. Proc. Natl. Acad. Sci. USA **75:** 1783–1787.

JOHNSON, A. D., C. O. PABO and R. T. SAUER, 1980 Bacteriophage λ repressor and Cro proteins: interactions with operator DNA. Methods Enzymol. **65:** 839–856.

KIM, J. G., Y. TAKEDA, B. W. MATTHEWS and W. F. ANDERSON, 1987 Kinetic studies on Cro repressor-operator DNA interaction. J. Mol. Biol. **196:** 149–158.

LEBRETON, B., P. V. PRASAD, M. JAYARAM and P. YOUDERIAN, 1988 Mutations that improve the binding of yeast FLP recombinase to its substrate. Genetics **118:** 393–400.

LEWIS, M., A. JEFFREY, J. WANG, R. LADNER, M. PTASHNE and C. O. PABO, 1983 Structure of the operator-binding domain of λ repressor: implications for DNA recognition and gene regulation. Cold Spring Harbor Symp. Quant. Biol. **47:** 435–440.

NELSON, H. C. M., and R. T. SAUER, 1985 Lambda repressor mitations that increase the affinity and specificity of operator binding. Cell **42:** 549–558.

OHLENDORF, D. H., W. F. ANDERSON, R. G. FISHER, Y. TAKEDA B. W. MATTHEWS, 1982 The molecular basis of DNA-protein recognition inferred from the structure of Cro repressor. Nature **298:** 718–723.

OHLENDORF, D. H., W. F. ANDERSON, M. LEWIS, C. O. PABO and B. W. MATTHEWS, 1983 Comparison of the structure of Cro and repressor proteins from bacteriophage λ. J. Mol. Biol. **169:** 757–769.

PAKULA, A. P., V. Y. YOUNG and R. T. SAUER, 1986 Bacteriophage lambda Cro mutations: effects on activity and intracellular degradation. Proc. Natl. Acad. Sci. USA **83:** 8829–8833.

PTASHNE, M., A. JEFFREY, A. D. JOHNSON, R. MAURER, B. J. MEYER, C. O. PABO, T. M. ROBERTS and R. T. SAUER, 1980 How the λ repressor and Cro work. Cell **19:** 1–11.

ROBERTS, T. M., H. SHIMATAKE, C. BRADY and M. ROSENBERG, 1977 Sequence of *cro* gene of bacteriophage lambda. Nature **270:** 274–275.

SADLER, J. R., H. SASMOR and J. L. BETZ, 1983 A perfectly symmetric *lac* operator binds the *lac* repressor very tightly. Proc. Natl. Acad. Sci. USA **80:** 6785–6789.

SARAI, A., and Y. TAKEDA, 1987 How Cro and λ repressor recognize operator DNA sequences. In *Protein Structure and Design*, Edited by D. OXENDER. Alan R. Liss, New York (in press).

TAKEDA, Y., A. FOLKMANIS and H. ECHOLS, 1977 Cro regulatory protein specified by bacteriophage λ. J. Biol. Chem. **252:** 6177–6183.

TAKEDA, Y., D. H. OHLENDORF, W. F. ANDERSON, and B. W. MATTHEWS, 1983 DNA-binding proteins. Science **221:** 1020–1026.

TAKEDA, Y., S. SARAI and V. M. RIVERA, 1988 Analysis of the sequence-specific interactions between Cro repressor and operator DNA by systematic base substitution mutations. Proc. Natl. Acad. Sci. USA (in press).

Communicating editor: G. MOSIG