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NH_2-terminal arm of phage λ repressor contributes energy and specificity to repressor binding and determines the effects of operator mutations

(NH_2-terminal deletions/protein overproduction/ATA initiation codon/front-side and back-side contacts)

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ABSTRACT **Several lines of evidence indicate that the phage λ repressor recognizes its operator by using, in part, an α helix (the "recognition helix"), which it inserts into the major groove of DNA. In addition to its recognition helix, λ repressor has an "arm," consisting of the first six amino acids, that wraps around the DNA helix. We constructed plasmids that, in *Escherichia coli*, direct the expression of derivatives of λ repressor that lack the NH_2-terminal one, three, six, or seven amino acids. We studied these modified proteins *in vivo* and *in vitro*, and from our results we argue that the arm: (*i*) contributes a large portion of the binding energy; (*ii*) helps to determine sequence specificity of binding and, in particular, the relative affinities for two wild-type binding sites; (*iii*) determines entirely repressor's response to one operator mutation (a "back-side" mutation); (*iv*) magnifies repressor's response to other operator mutations ("front-side" mutations); and (*v*) increases the sensitivity of repressor binding to salt concentration and temperature.**

Models based upon x-ray crystallographic studies (1–3) and upon sequence homologies (4, 5) suggest that many prokaryotic regulatory proteins recognize their binding sites by a common mechanism. According to these models (6–8), such proteins make sequence-specific contacts by inserting an α helix, the "recognition helix," into the major groove of DNA. In one case it has been shown that replacing the recognition helix from one protein with the recognition helix of another alters the binding specificity (9). Phage λ repressor is unusual because, in addition to a recognition helix, it also bears a flexible NH_2-terminal "arm" that consists of the first six amino acids and evidently wraps around the DNA (10). According to the current picture (6), each monomer of a repressor dimer inserts a recognition helix into the major groove on one face of the DNA helix, the "front side." The two arms of the dimer wrap around the DNA, making specific contacts in the major groove on the opposite face, the "back side," and also making nonspecific contacts to the DNA phosphates. Phage λ Cro protein, which lacks an arm, binds to the same operator sites as does repressor. Cro also binds as a dimer, inserting a recognition helix from each monomer into the major groove on the front side of the DNA helix, but does not contact the back side of the operator (7).

Previous experiments have shown that phage λ repressor derivatives with defects in the arm have a reduced affinity for operator. A point mutation changing Lys-4 to Gln-4 greatly reduced repressor binding (11). Proteolytic removal of the NH_2-terminal three amino acid residues decreased the affinity of a repressor fragment [the NH_2-terminal domain (12)] for an operator site and altered the ability of the same repressor fragment to protect guanines in the operator from methylation by dimethyl sulfate (10). Since removal of the first three amino acids does not change the global conformation of the protein (13), alterations made to the arm are unlikely to affect the binding of the recognition helix.

To examine more fully the role of the arm in operator recognition by phage λ repressor, we constructed repressor genes encoding proteins missing all or part of the NH_2-terminal arm. We expressed the truncated proteins in *E. coli*, studied their properties *in vivo*, and examined the binding of the purified proteins *in vitro*. We find that the arm is required for DNA binding, enhances the ability of phage λ repressor to discriminate between two wild-type operator sites (O_R1 and O_R3), and contributes to the sensitivity of the binding reaction to salt concentration and temperature. Repressor and Cro respond differently to operator mutations (refs. 14–16 and unpublished data), and our experiments indicate that the arm is responsible for this difference in at least three cases. Two of these operator mutations affect base pairs not contacted by the arm (6), yet the arm is partly responsible for their effect on repressor binding. We discuss the likely mechanisms for these effects.

MATERIALS AND METHODS

Enzymes and Reagents. Reagents were purchased from Millipore (DNase I), Boehringer Mannheim (*Cla* I), New England Biolabs (other restriction enzymes) New England Nuclear (T4 DNA ligase and [α^{32}P]dNTPs), Bethesda Research Laboratories (BAL-31), and P-L Biochemicals (mung bean nuclease).

Protein Purifications. Wild-type repressor (R_{1-236}) was prepared by S. Munroe from RB791/pEA305 (17) as described (18). Derivatives lacking the first one or three residues of repressor (R_{2-236} and R_{4-236}) were purified by the same method. R_{7-236} was purified by precipitation with polyethylene imine and chromatography on DEAE-cellulose, carboxymethyl-Sephadex, Affi-Gel blue, hydroxyapatite, and Sephadex G-100 superfine. The proteins were obtained 98–99% pure (95% for R_{7-236}) as judged by gel electrophoresis (not shown).

Other Procedures. Restriction fragments were labeled at the 3′ end (19) and isolated (20) as described. DNase I protections were performed as described (21) except that the KCl concentration and temperature were varied and 8% polyacrylamide gels were used. Dimethyl sulfate protections were performed as described (22, 23). Gel electrophoresis of proteins was performed on 15% polyacrylamide gels by the method of Laemmli (24). The activity of R_{1-236} and R_{2-236} was determined as described (18). Unlike R_{1-236} and R_{2-236}, R_{4-236} does not retain operator DNA on a nitrocellulose filter, so its activity was not determined. NMR spectroscopy was performed as described (25). DNA sequencing was performed by the method of Maxam and Gilbert (20).

Strains. The *E. coli* strain RB791 [$lacI^QL8$] was obtained

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from R. Brent (26). NK5031(λ200) contains a *lacZ* gene transcribed from the phage λ promoter P_R, and is described by Meyer *et al.* (27). λ phages λKH54 [λcI$^-$] (28), λ*v2v3* (29), the virulent phage λ*vir* [λ*v2v1v3*] (29), and the supervirulent

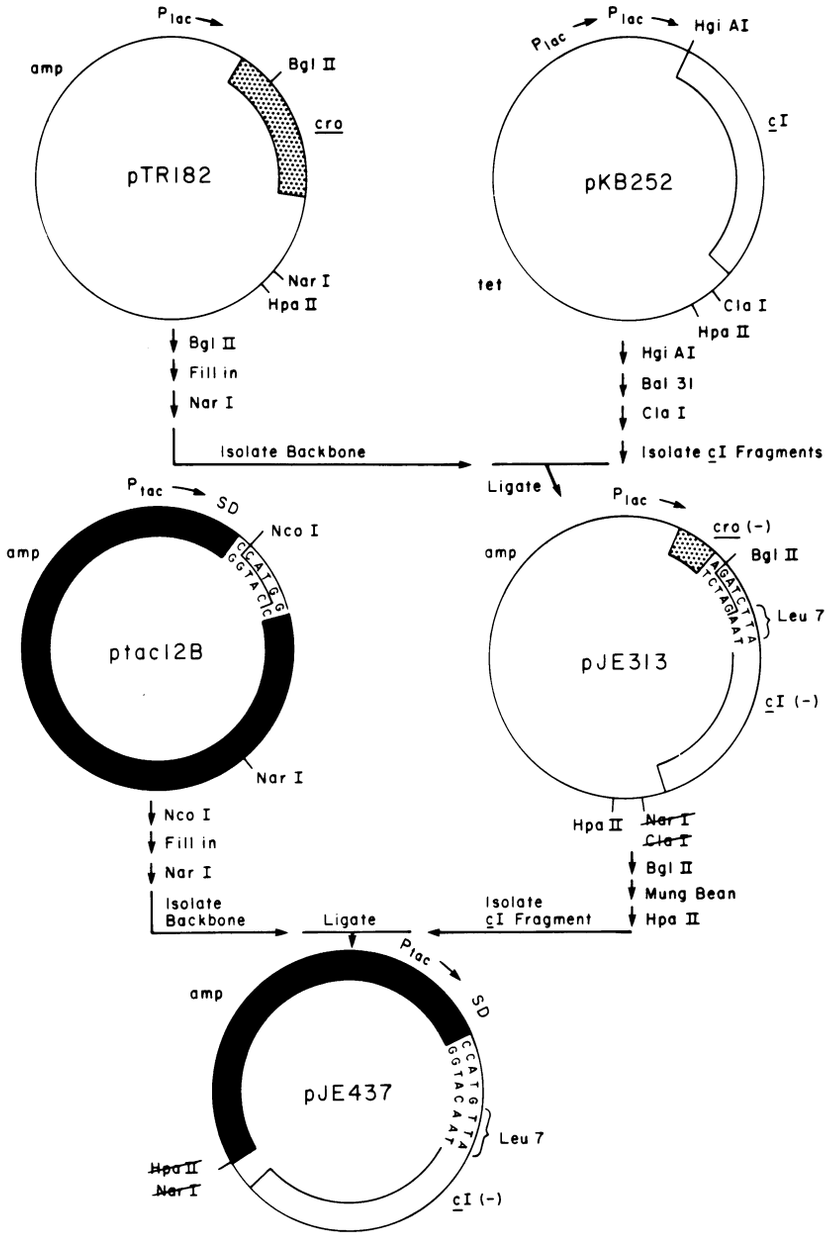

FIG. 1. Construction of plasmids that overproduce phage λ repressor derivatives having NH_2-terminal deletions. pJE437, encoding R_{7-236}, was constructed in two steps. First, the desired deletion was constructed by using one fragment from pTR182 (30) and one from pKB252 (31). pTR182 was digested at the unique *Bgl* II site lying within the *cro* gene; the ends were made flush with the large fragment of *E. coli* DNA polymerase I and the four dNTPs; the flush-ended linear plasmid was digested with *Nar* I; the large fragment containing the *amp* gene and the 5′ end of the *cro* gene was isolated by gel electrophoresis. pKB252 was digested with *Hgi*AI; the fragments were treated with exonuclease BAL-31 for various times between 10 and 100 sec, and the time points were pooled; the mixture was digested with *Cla* I, and fragments containing various amounts of the *c*I gene were isolated by gel electrophoresis. These fragments were ligated to the pTR182 fragment (*Nar* I and *Cla* I generate identical sticky ends). Plasmids that incorporated a *c*I fragment of the proper length (i.e., beginning at the Leu-7 codon TTA) or a fragment that was 1 base pair too short regenerated the *Bgl* II site. The proper deletion was distinguished from the deletion removing 1 base pair too many by a simple biological test. λ*v2v3* makes normal turbid plaques on cells containing plasmids bearing the correct deletion but makes clear plaques on cells containing plasmids with the incorrect deletion. The incorrect deletion creates an in-frame Cro–repressor fusion protein that prevents lysogeny presumably by sequestering the repressor made by the λ*v2v3* phage in inactive mixed dimers. A plasmid that contained a *Bgl* II site and did not cause λ*v2v3* to plate clear was called pJE313. DNA sequencing confirmed the deletion endpoint. The DNA encoding amino acids 7–236 of repressor was next ligated to an expression vector. ptac12B (25) contains the strong *tac* promoter (17, 32) and the *lac* Shine–Dalgarno (SD) sequence, followed by an *Nco* I site. A fragment of ptac12B was prepared as the pTR182 fragment above. The flush end of this fragment ends with an ATG, which serves as the initiation codon for the truncated *c*I genes. pJE313 was digested with *Bgl* II; the sticky end was removed with mung bean nuclease; the flush-ended linear plasmid was digested with *Hpa* II, and the *c*I-containing fragment was purified. The two fragments were ligated to give pJE437. The other deletions were constructed by a streamlined version of the above procedure. *c*I-containing fragments were prepared from pKB252 as above, except *Hpa* II was used in place of *Cla* I. They were ligated to the same ptac12B fragment used to make pJE437. The resulting plasmids, including pJE437, were used to transform RB791. Plasmids carrying the initiation codon in the same reading frame as the *c*I gene conferred immunity to λcI$^-$ and/or directed the production of a large amount of a 26,000-dalton protein. Restriction analysis established that the plasmids had the expected structures.

phage λ*4v* [λ*v2v305v3cvs326*] (14) were from this lab's strain collection.

RESULTS

Truncated Repressor Proteins Can Be Overproduced *in Vivo*. To produce large quantities of phage λ repressor derivatives that lack NH_2-terminal residues, we deleted DNA from the 5′ end of the repressor gene (*cI*) and fused the shortened genes to a vector containing three control signals: a strong promoter derived from the *lac* promoter, a Shine–Dalgarno sequence, and a translation initiation codon (ATG) (see Fig. 1). The DNA sequence of 90 base pairs centered on the initiation codon was determined for five plasmids. These plasmids encode R_{2-236}, R_{4-236}, R_{7-236}, and R_{8-236} (see Fig. 2).

The *tac* promoter driving the repressor gene on each of these plasmids is controlled by the *lac* repressor; in an appropriate host strain, the expression of the truncated repressors can be induced by the addition of isopropyl thiogalactopyranoside to the culture. From gel electrophoresis (not shown), we estimate that R_{4-236} and R_{7-236} constitute 2% of the cell's protein in the absence of isopropyl thiogalactopyranoside and 20% of the cell's protein in the presence of 1 mM isopropyl thiogalactopyranoside. The same levels are produced by a similar plasmid encoding wild-type repressor (17). For unknown reasons, R_{2-236} constitutes 1% of the cell's protein in the presence or absence of isopropyl thiogalactopyranoside. We isolated a second plasmid that encodes R_{4-236} in which the initiator codon ATG is replaced by ATA. In fully induced cells bearing this plasmid, R_{4-236} constituted less than 0.5% of the cell's protein. Repressor constituted approximately 0.01% of the cell's protein in a lysogen.

The NH_2-terminal protein sequence of R_{2-236} is Thr-Lys-Lys-Lys..., as predicted, and fewer than 5% of the molecules retained the methionine (W. Lane, personal communication). In our preparation of purified R_{4-236}, 80% of the molecules retained an unblocked initiator methionine and the remaining 20% began with Lys-4 (25). The NH_2-terminal protein sequence of R_{7-236} was not determined. NMR spectroscopy, which can detect minor conformational changes in mutant phage λ repressors (33), showed that purified R_{2-236}, R_{4-236}, and R_{7-236} are all properly folded (not shown).

Removal of the Arm of λ Repressor Abolishes DNA Binding. *In vivo*, wild-type repressor, at the level found in a lysogen, confers immunity to infection by λ*cI*$^-$ and represses transcription of a P_R-*lacZ* fusion to less than 1/100th by binding to the operator sites O_R1 and O_R2 (27). In contrast, R_{7-236}, when provided by a plasmid at 150 times the level found in a lysogen, failed to confer immunity to λ*cI*$^-$ and failed to repress transcription of the same P_R-*lacZ* fusion (not shown). *In vitro*, DNase I protection experiments showed specific protection of the operators by R_{1-236} but not by R_{7-236}, even at a concentration of R_{7-236} that was 8000-fold higher than the R_{1-236} concentration sufficient to observe protection (not shown).

R_{8-236} did not confer immunity to λ*cI*$^-$ either. It was not studied further.

Removal of the NH_2-Terminal Three Amino Acids Reduces Binding Affinity. The following *in vitro* and *in vivo* experiments showed that R_{2-236} has an affinity for operator indistinguishable from that of wild-type repressor and that R_{4-236} has a lower, but still substantial, affinity. *In vitro*, purified R_{1-236}, R_{2-236}, and R_{4-236} specifically protected the operators from DNase I. The same concentrations of R_{2-236} as R_{1-236} were required to protect the operators, but roughly 30-fold more R_{4-236} was needed to observe the same protection. One measure of the activity of repressors *in vivo* is the immunity conferred against infection by λ phages. We made use of three tester phages that increase in their resistance to repression in the order λ*cI*$^-$<λ*vir*<λ*4v*. λ*4v* grew on cells in which 2% of the protein was R_{4-236}, but it did not grow on cells containing similar levels of R_{1-236} or R_{2-236}. λ*vir* and λ*cI*$^-$ did not grow on cells containing this concentration of any of these proteins.

The Arm Contacts the Back Side of the Operator. Repressor binds primarily to one side of the DNA helix (6). The ethylation of some phosphates inhibits repressor binding, and all these phosphates lie on one side of the operator (15, 34), which we call the "front side" (10). However, repressor protects the N-7 atoms of guanines from methylation by dimethyl sulfate on both the front and back sides (10, 23). Inspection of a model of operator DNA (not shown) shows which guanines expose their N-7 atoms on the front side of the operator. The results of our dimethyl sulfate protection experiments are shown in Fig. 3. Guanines on the front side were protected by R_{1-236}, R_{2-236}, and R_{4-236}, while guanines on the back side were protected by R_{1-236} and R_{2-236} only. The only exceptions were three guanines not protected by any of the three proteins.

R_{4-92}, a proteolytic fragment of wild-type repressor, protects the same guanines in O_R1 as does R_{4-236} (10). Cro protein, which lacks an arm, also fails to protect back-side guanine N-7s from dimethyl sulfate (23).

The Arm Partially Determines Specificity and the Effect of Operator Mutations. Phage λ repressor binds to O_R1 more tightly than to O_R3, while Cro binds with the opposite order of affinity (21), a fact that is crucial for λ gene regulation (34). We show here that the arm is partly responsible for this difference. R_{1-236} and R_{4-236} both bound more tightly to O_R1 than to O_R3, but this difference was 3-fold more for R_{1-236} than for R_{4-236} (see Table 1, lines 1 and 2). R_{4-236} bound less tightly than R_{1-236} to both O_R1 and O_R3, but the loss of the first three residues reduced the binding affinity for O_R1 more than that for O_R3.

The operator mutation *v3* removed a putative arm contact

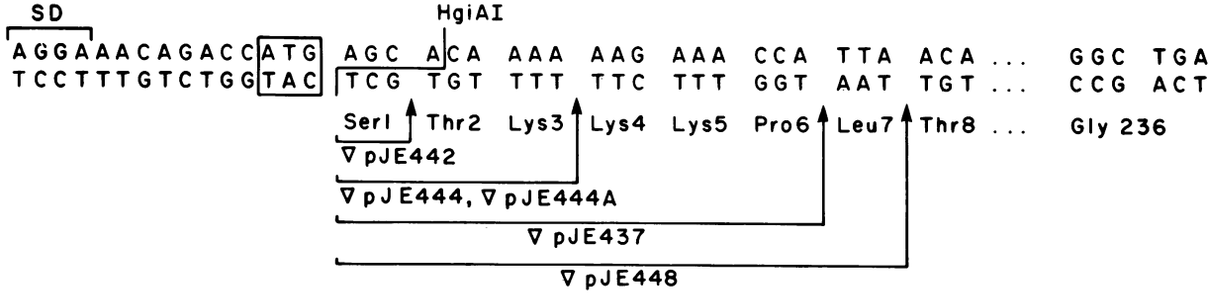

FIG. 2. Sequence of NH_2-terminal deletions. The sequence of the NH_2-terminal end of phage λ repressor and the first few base pairs of the *cI* gene are shown. The sequence to the left of the ATG derives from ptac12B. The brackets denote the base pairs removed in the deletions. pJE442, pJE444, pJE444A, pJE437, and pJE448 encode R_{2-236}, R_{4-236}, R_{4-236}, R_{7-236}, and R_{8-236}, respectively. pJE444A is identical to pJE444 except that the translation initiation codon ATG is replaced by ATA.

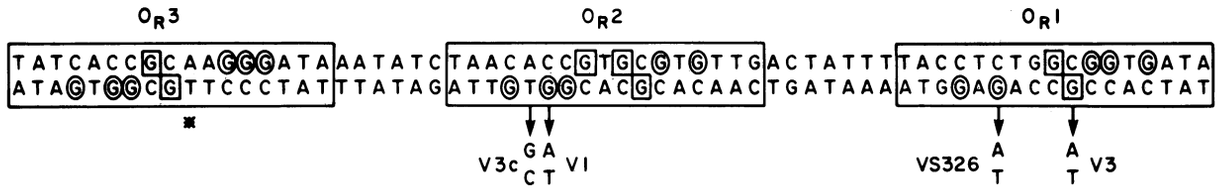

FIG. 3. Dimethyl sulfate protection of λO_R. O_R1, O_R2, and O_R3, the three binding sites for phage λ repressor and λ Cro protein, are boxed. The N-7 position of circled guanines is protected from dimethyl sulfate by R_{1-236}, R_{2-236}, and R_{4-236}. Squares denote guanines that are protected by R_{1-236} and R_{2-236} only. Our results differ from previous experiments (23, 35) in that we see a clear protection of the guanine at position 9 of O_R3. The O_R mutations mentioned in the text are shown with their base-pair changes (14). The asterisk marks the base pair in O_R3 that is analogous to the mutant base pair in O_R1v3.

from the back side of O_R1 by replacing a G·C base pair at position 8 with a T·A base pair. O_R3, as well as O_R1v3, has a T·A base pair at this position (see Fig. 3). *v3* reduced the affinity of R_{1-236} for O_R1 to at most 1/20th but did not affect R_{4-236} binding (Table 1, line 2 vs. line 4) or Cro binding (not shown). This result, combined with the dimethyl sulfate protection experiments, argues that R_{1-236}, but not Cro or R_{4-236}, makes a sequence-specific contact to position 8 of the operator, on the back side of the DNA helix.

The following experiments show that the arm also influences the repressor's response to mutations that remove front-side contacts. The mutations *vs326* and *v1* removed predicted contacts for repressor from the front side of O_R1 and O_R2, respectively. Each of these mutations replaced a G·C base pair at position 6 with a T·A base pair (see Fig. 3). The reduction of R_{1-236} binding caused by these mutations was 10- to 50-fold larger than the reduction of R_{4-236} binding (Table 1, lines 2 and 3 vs. line 1). The current model (6) proposes that the contacts between repressor and the base pair at position 6 are made by the recognition helix and not by the arm. In the discussion we suggest an explanation for the role of the arm in determining the magnitude of the effect of these mutations.

The Arm Affects the Sensitivity of Binding to Salt and Temperature. Alterations of either repressor or operator that prevented correct binding of the arm to the back side of the DNA helix reduced the sensitivity of repressor binding to increased salt concentration and temperature. R_{1-236} binding to O_R1^+ was more sensitive to salt and temperature than was R_{4-236} binding (see Table 2). The functional groups exposed in the major groove on the back side of O_R1v3 were similar to those on the back side of O_R3, and the functional groups on the front side of O_R1v3 were identical to those of O_R1. The binding of R_{1-236} to O_R1v3 and O_R3 was less sensitive to salt and temperature than was binding to O_R1 (see Table 2), suggesting that the salt and temperature effects are primarily due to interactions between the arm and the back side of the operator.

Table 1. Binding of R_{1-236} and R_{4-236} to various operator sites

DNA			R_{1-236}			R_{4-236}		
O_R1	O_R2	O_R3	O_R1	O_R2	O_R3	O_R1	O_R2	O_R3
+	+	+	<1	<1	100	1	1	30
+	*v1*	+	<1	8000	140	1	150	50
vs326	+	+	500	15	15	60	7	7
v3	*v1*	+	20	7000	120	1	>100	30

Values indicate the relative concentrations of repressor dimers required for half-maximal protection of the indicated operator site in a DNase I protection experiment. For R_{1-236}, a dimer concentration of 0.05 nM was set equal to 1 and all numbers were normalized to this value. For R_{4-236}, all numbers were normalized to a value of 5 nM. The concentration of dimers was calculated from the known concentration of repressor polypeptides by assuming a dimerization constant of 2×10^{-8} M (36). All experiments were done at 4°C in 50 mM KCl. The concentrations are accurate measures of affinity only where it can be assumed that the majority of repressor dimers are not bound to DNA at equilibrium (21). Under the conditions used in these experiments, this assumption is not valid for the case of R_{1-236} binding to O_R1^+ because the binding is too tight. For this case, the actual affinity is not given by the observed concentration at half-maximal protection (0.05 nM) but by a lower concentration.

DISCUSSION

Our experiments have confirmed and extended the results obtained with the first "armless λ repressor" (10), a proteolytic fragment of repressor consisting of amino acids 4–92. Unlike R_{4-92}, our repressor derivatives dimerize efficiently since they retain the COOH-terminal domain (12). We have used these derivatives to show that the arm contributes to the affinity, specificity, and sensitivity to salt concentration and temperature of repressor binding.

The arm provides a large part of the binding energy of phage λ repressor. Removal of the NH_2-terminal three residues reduces the binding affinity of repressor to roughly 1/30th, and removal of the whole arm (six residues) reduces the affinity by at least 3 orders of magnitude. Neither deletion unfolds the protein.

Dimethyl sulfate protection experiments show that the arm contacts the back side of the operator. We have shown that these contacts help to determine the sequence specificity of repressor binding. The *v3* mutation removes a putative arm contact site, and the inhibitory effect of *v3* on repressor binding is dependent on the presence of the arm. O_R3, like O_R1v3, lacks a guanine at position 8. The ratio of binding affinities of O_R1 to O_R3 is larger for R_{1-236} than for R_{4-236} presumably because the absence of this guanine in O_R3 adversely affects R_{1-236} binding but not R_{4-236} binding. Dimethyl sulfate protections, model building, and the effect on repressor binding of the absence of guanine at position 8 are

Table 2. Salt and temperature sensitivity of repressor binding

Repressor binding	Temperature, °C	KCl 50 mM	KCl 200 mM
R_{1-236} binding to O_R1^+,	4	<1	20
normalized 1 = 0.05 nM	37	20	1200
R_{4-236} binding to O_R1^+,	4	1	100
normalized 1 = 5 nM	37	20	200
R_{1-236} binding to O_R1v3,	4	1	110
normalized 1 = 1 nM	37	9	180
R_{1-236} binding to O_R3^+,	4	1	70
normalized 1 = 6 nM	37	4	75

The numbers are relative concentrations of protein dimers required for half-maximal protection of the indicated operator site in DNase I protection experiments. All of the values in each set of four experiments have been normalized to the dimer concentrations shown to the left of each set. All experiments were done on O_R2^- templates to eliminate cooperative binding (21).

all consistent with the idea that the arm directly contacts the N-7 atom of guanine at position 8. Also consistent with this are the facts that Cro, which lacks an arm, does not protect guanines at position 8 and Cro binding is not affected by the *v3* mutation.

Operator mutations affecting repressor–operator contacts made on the "front side" of both O_R1 and O_R2 reduce R_{1-236} binding much more than R_{4-236} binding, yet the arm is not predicted to bind to this side of the operator (6). This result suggests an explanation for the different effects that one such mutation, *vs326* (position 6 of O_R1), has on repressor and Cro binding. The models for repressor and Cro binding propose that they make similar bidentate hydrogen bonds to the guanine at position 6 (6, 7), yet *vs326* reduces repressor binding to 1/150th and Cro binding to only 1/5th (15). We propose that much of this differential effect is due to the failure of the arm to bind properly when repressor binds to O_R1*vs326*. We imagine that when a repressor dimer binds to an operator, the two arms can reach their proper contact sites on the back side of the DNA only if the recognition helices can be correctly positioned in the major groove. If an amino acid residue cannot make its normal contact on the front side because of an operator mutation, the recognition helix may have to move away from the DNA to accommodate the steric block, thereby pulling the arm out of reach of its contact site. According to this idea, *vs326*, and similarly *v1*, affects R_{1-236} binding more than R_{4-236} or Cro binding because the operator mutations cause R_{1-236} to lose both front-side and back-side contacts and cause R_{4-236} or Cro to lose only front-side contacts.

Removing the first three residues from repressor or changing a base pair contacted by the arm results in a lower sensitivity of repressor binding to temperature. This implies that R_{4-236} has a lower enthalpy (ΔH) of binding than that of R_{1-236} (37). Some of the favorable enthalpy of binding due to interaction of the arm is likely to be due to ionic interactions, most plausibly between the lysines in the arm and the DNA phosphates, since truncation of the arm or mutation of a contact site reduces the sensitivity of binding to salt (38).

Phage λ Cro protein does not wrap around the DNA and binds well to the same operator sites as repressor. Why is an arm dispensable for Cro but not for repressor? The models for Cro and repressor binding (6, 7) show more contacts to operator DNA for Cro than for residues 7–236 of repressor (the arm is not included in the repressor model). It seems likely that the recognition helix of repressor does not provide sufficient binding energy or specificity and that repressor needs the arm to compensate.

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