Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7510–7514, August 1995
Biochemistry

Operator binding by λ repressor heterodimers with one or two N-terminal arms

YONG-IN KIM* AND JAMES C. HU*†‡

*Department of Biochemistry and Biophysics and †Center for Macromolecular Design, Institute for Biosciences and Technology, Texas A&M University, College Station, TX 77843-2128

Communicated by Mark Ptashne, Harvard University, Cambridge, MA, May 12, 1995

ABSTRACT **The first 6 amino acids (NH_2-Ser[1]-Thr[2]-Lys[3]-Lys[4]-Lys[5]-Pro[6]) of bacteriophage λ *c*I repressor form a flexible arm that wraps around the operator DNA. Homodimeric λ repressor has two arms. To determine whether both arms are necessary or only one arm is sufficient for operator binding, we constructed heterodimeric repressors with two, one, or no arms by fusing the DNA binding domain of λ repressor to leucine zippers from Fos and Jun. Although only one arm is visible in the cocrystal structure of the N-domain–operator complex, our results indicate that both arms are required for optimal operator binding and normal site discrimination.**

Bacteriophage λ *c*I repressor uses two DNA binding motifs to recognize operator DNA: residues 33–51 of each monomer contain a helix–turn–helix motif (1) that lies in the major groove of each half of a pseudosymmetric operator and residues 1–6 form a flexible arm in solution (2) that wraps around the operator DNA to contact bases and phosphates on the back side of the operator (3). Although genetic and biochemical studies have shown that removing the N-terminal arms of λ repressor destroys operator binding (3–5), these studies do not distinguish between the roles of the two arms in the homodimeric wild-type protein. In cocrystal structures of the repressor–operator complex solved to 1.8-Å resolution at −15°C, electron density for only one of the two arms is observed (5, 6). The visible arm lies in the consensus half site of the O_L1 oligodeoxynucleotide used in the crystals and makes contacts with both specific bases and the phosphate backbone in the DNA. Bases in the consensus half site that contact the amino acids of the arm are protected from chemical modification (7) and contribute to the binding energy of complex formation, as determined by the effects of operator mutations on repressor binding (8). The lack of electron density from the arm of the repressor monomer bound to the nonconsensus half site is presumably due to thermal motion and/or conformational heterogeneity within the crystal. Equivalent bases in the nonconsensus half site of O_R1 are not protected (7) and mutations in these positions have relatively modest effects on repressor binding (8).

These results suggest that the two arms have different roles in the repressor–operator complex. Clearly, the arm bound to the consensus half site makes several energetically important contacts to DNA. In contrast, the role of the arm in the monomer bound to the nonconsensus half site is unclear. The nonconsensus arm might assume several different conformations that collectively contribute binding energy from contacts between basic amino acid side chains and phosphates in the operator DNA. On the other hand, the nonconsensus arm might be a remnant that is present only as a consequence of repressor being a homodimer. These possibilities cannot be distinguished by examining the effects of mutations in the arm in the context of wild-type λ repressor, since such mutations necessarily alter both the consensus and nonconsensus arms in homodimeric *c*I protein.

In this report we describe experiments to distinguish the functional roles of the two arms in λ repressor, by examining the DNA binding properties of a series of heterodimeric repressor–leucine zipper fusion proteins with zero, one, or two arms. Although only one of the two arms can be visualized in the cocrystal structure, we find that both arms are necessary for binding to high-affinity operators and for discrimination among different operator sequences.

MATERIALS AND METHODS

Strains and Plasmids. Repressor fusions were expressed from compatible plasmids in *Escherichia coli* AG1688 (9). Fusions with the intact N-domain were constructed in pJH391 (9), and fusions lacking the N-terminal arm were constructed in pHH7005 (H. Hunter and J.C.H., unpublished data), a plasmid similar to pJH370 (10) except that it contains a unique *Xba* I site engineered upstream of the translation start of the *c*I gene and an amber mutation at Trp[129] (H. Hunter and J.C.H., unpublished data). The amber mutation was removed in subsequent cloning steps. Plasmids derived from pACYC184 were constructed from pJH541, in which the *tet* gene from pACYC184 is replaced by the *tet* gene of pSELECT-1 from Promega. This removes the *Sal* I and *Bam*HI sites from the *tet* gene. An *Eco*RI–*Eco*RV fragment from pJH370 containing wild-type repressor–GCN4 fusion was cloned into pJH541 between the *Eco*RI site and the *Pvu* II site at position 515 on the pACYC184 map to generate pJH550. Fusion constructs described in this report were generated by replacing the leucine zipper, the repressor fragment, or both in pJH550.

To remove the N-terminal arm, an in-frame deletion of residues 1–6 was generated by PCR and used to replace the equivalent segment in pHH7005. Where indicated, the gene encoding the armless repressor domain was fused to Fos and Jun leucine zippers by reconstruction of similar plasmids. Expression of repressor fusions with deletions of residues 1–6 required addition of an N-terminal methionine residue before Leu[7]. The arm deletion does not appear to affect the synthesis or stability of λ repressor *in vivo* based on the accumulation of protein upon induction with isopropyl β-D-thiogalactoside. Circular dichroism spectra of purified proteins (see below) show similar helical content in the presence or absence of the arm (data not shown). We conclude that the global folding is not changed in the mutant proteins with the arm residues deleted.

Oligonucleotide cassettes that encode the Fos and Jun leucine zippers were constructed by mutual primed synthesis and cloned between the *Sal* I and *Bam*HI sites of the *c*I fusion vectors in the presence or absence of the Δ1–6 mutation to generate in-frame fusions after residue 132 of repressor (11). Amino acid sequences used for the leucine zippers were as

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

‡To whom reprint requests should be addressed.

follows: Fos, LTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAAR; Jun, HMRRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKY. For purification, identical leucine-zipper sequences were used with the addition of the sequence GGHHHHHH to the C-terminal end. Addition of the histidine tag did not affect the activity of the fusion proteins *in vivo* (data not shown).

Repressor Activity *in Vivo*. Binding to λ operators *in vivo* was tested by examining sensitivity to the cI^- phage λKH54 in a cross-streak assay (12) and by measuring β-galactosidase expression from a λ O_R2^- P_R–*lacZ* fusion present on the λ202 prophage (13) in strain JH372 (10). β-Galactosidase was measured by the $CHCl_3$/SDS lysis method of Miller (14) from logarithmic-phase cultures grown in M9 glucose minimal medium supplemented with Casamino acids. Repression of *lacZ* expression was calculated as 1 − (β-galactosidase activity in strain X/β-galactosidase activity in control strain) and is given as the average of at least two experiments.

Purification of Fusion Proteins. Fusion proteins with six histidine residues fused at the C-terminal ends of the Fos and Jun leucine zippers were purified from transformants of *E. coli* AG1688 grown in antibiotic-supplemented LB broth or 2xYT/2% (wt/vol) glucose and induced with isopropyl β-D-thiogalactoside added to 3 mM when the cells reached an OD_{600} of 0.5–0.7. Cells were harvested after 5 hr of induction and resuspended in sonication buffer [10 mM Tris·HCl, pH 7.9/500 mM NaCl/0.1 mM phenylmethylsulfonyl fluoride/30% (vol/vol) glycerol] containing lysozyme (1 mg/ml). After 30 min on ice, lysates were sonicated and centrifuged for 20 min at 17,000 × *g*. The supernatant was added to 4 ml of nickel nitrilotriacetate resin (Qiagen, Chatsworth, CA) equilibrated with buffer D (20 mM Tris·HCl, pH 7.9/100 mM KCl/0.1 mM phenylmethylsulfonyl fluoride/30% glycerol). The resin was packed into a column and washed by step elution with 10–20 bed volumes of buffer D containing imidazole at 10 mM, 40 mM, 80 mM, and 500 mM. The fusion proteins were eluted at 80 mM imidazole [cI^+–Fos and $cI(\Delta1–6)$–Fos proteins], or 0.5 M imidazole [cI^+–Jun and $cI(\Delta1–6)$–Jun proteins]. Fractions containing each fusion protein were pooled, dialyzed against storage buffer (20 mM Tris·HCl, pH 7.9/100 mM KCl/30% glycerol), and stored at −20°C. Total active monomer concentrations were calculated from the stoichiometry experiments as described by Riggs *et al.* (15). Purified cI^+–Jun protein was 77% active, and cI^+–Fos protein was 52% active.

Repressor Activity *in Vitro*. Quantitative DNase I footprint titration experiments and determination of fractional occupancies of individual sites were done as described by Brenowitz *et al.* (16), with slight modifications. Operator DNA fragments were prepared by labeling *Bgl* II-digested DNA from pKB252 (17) with [α^{32}P]dGTP by DNA synthesis catalyzed by the Klenow fragment of DNA polymerase I. End-labeled DNA was redigested with *Nsi* I, and a 338-bp fragment was purified. Binding experiments were performed in 10 mM Tris·HCl, pH 7.0/200 mM KCl/2.5 mM $MgCl_2$/1 mM $CaCl_2$/bovine serum albumin (100 μg/ml)/0.1 mM EDTA/sonicated salmon sperm DNA (2 μg/ml) at 20°C. Reaction mixtures were incubated for 30–60 min prior to addition of operator DNA to allow equilibration of protein homodimers and heterodimers. DNA fragments were added and mixtures were incubated an additional 30 min to allow DNA binding to come to equilibrium. Equilibrium mixtures contained 15,000–20,000 cpm of ^{32}P-labeled operator DNA in 100 μl (≤10 pM operator, final concentration). DNase I (5 ng) was added in 5 μl and incubated for 1 min at 20°C. DNase I digests were resolved on 6% polyacrylamide/urea denaturing gels, and the amount of label in individual bands was quantitated by using a Fuji (Fuji Medical Systems, Stamford, CT) model BAS2000 phosphor imaging system at the Gene Technologies Laboratory in the Department of Biology at Texas A&M University.

Theoretical binding curves were calculated by using the program KALAIDEGRAPH by fitting data to a coupled dimerization-DNA binding reaction $2R + O \rightleftharpoons R_2 + O \rightleftharpoons R_2O$, where R represents repressor and O represents operator DNA. For this equilibrium, formation of the repressor–operator complex as a function of total protein is described by two equilibrium dissociation constants. $K_1 = [R]^2/[R_2]$ gives the proportions of repressor monomers and dimers, and $K_2 = [R_2][O]/[R_2O]$ describes the intrinsic binding of dimers to operator DNA. These two equilibrium dissociation constants are infinitely correlated and, therefore, cannot be determined without an independent measurement of one or the other half reaction. As this was not feasible, especially for experiments involving mixed dimer species, we generated approximate theoretical binding curves by fixing either K_2 (in Fig. 2) or K_1 (in Fig. 3) and by using nonlinear least squares regression analyses to evaluate the unfixed dissociation constant. It is important to note that slightly different binding curves with equally reasonable fits to the data can be generated by using different values for the fixed dissociation constant; these curves should be viewed as giving qualitative rather than quantitative information about the binding of repressor fusions.

RESULTS

To address whether both arms of λ repressor are necessary or only one arm is sufficient for operator binding, we constructed cells expressing heterodimeric repressors with no arms, one arm, or two arms. We exploited the fact that the dimerization specificity of repressor is largely controlled by its independently folding C-terminal domain (residues 133–236) (7), which can be replaced by a heterologous dimerization domain (10). To prepare repressor populations that were predominantly heterodimers, we fused the DNA binding domain of repressor to leucine zippers from the mammalian oncogenes Fos and Jun; these have been shown to preferentially form heterodimers (18). By coexpressing *c*I–Fos and *c*I–Jun fusions with and without N-terminal arms from compatible plasmids in *E. coli*, we could examine the effect of removing arms from repressor dimers one at a time (Fig. 1).

Table 1 shows how different individual repressor fusions used in this study bind operator DNA in *E. coli* as assayed by superinfection immunity and repression of a λ O_R2^- P_R–*lacZ* fusion (20). Replacement of the C-domain of repressor by leucine zippers from either Jun or GCN4, which can form homodimers, allows high-affinity binding to operator DNA. We used a deletion of residues 1–6 of repressor to remove the arms from the repressor domains. As has been previously shown for intact repressor, removing both N-terminal arms (Δ1–6) from the homodimeric Jun fusion prevents repression of either superinfecting phage or *lacZ* expression. Fusions to the Fos leucine zipper bind DNA poorly in either the presence or absence of the arms because Fos forms dimers only weakly

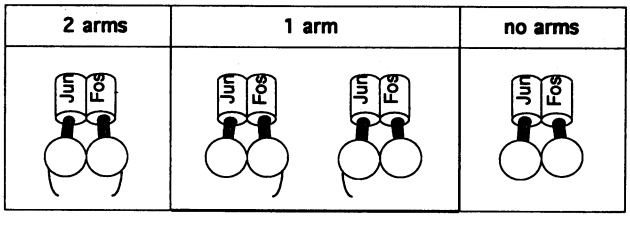

FIG. 1. Experimental scheme for testing the effect of removing N-terminal arms one at a time. Coexpression of *c*I–Fos and *c*I–Jun fusion proteins allows formation of a population of repressor molecules in which heterodimers predominate. Each leucine zipper can be fused to the intact N-terminal domain or to an allele in which the residues of the N-terminal arm have been deleted.

Table 1. Repression by fusions to the λ repressor DNA binding domain

Repressor domain	Leucine zipper	Immunity	% *lacZ* repression
None	None	Sens.	—
+	None	Sens.	36 ± 10
+	GCN4	Imm.	86 ± 3
+	Jun	Imm.	98 ± 1
Δ1–6	Jun	Sens.	3 ± 3
+	Fos	Sens.	38 ± 4
Δ1–6	Fos	Sens.	ND

Plasmids expressing fusion proteins with the indicated domains were introduced into strain JH372 [=AG1688(λ202)]. Operator binding *in vivo* was tested by two methods: (*i*) whether cells were sensitive or immune to killing by λKH54 (19) and (*ii*) inhibition of *lacZ* expression from the $O_R2^-O_R1^+$ P_R–*lacZ* fusion encoded by λ202 (20). ND, not determined. Data for percent *lacZ* repression are the mean ± SEM.

under physiological conditions and dimerization and DNA binding are coupled.

The *c*I–Jun fusions were cloned and expressed from a pACYC184-derived vector that confers tetracycline resistance, while *c*I–Fos fusions were expressed from a compatible ampicillin-resistant pBR322 derivative. These plasmids were cotransformed into *E. coli* in different combinations to generate strains expressing both *c*I–Fos and *c*I–Jun fusions. These different combinations of Fos and Jun fusions should form heterodimers with different numbers of arms.

Results for binding λ operators in *E. coli* by different combinations of repressor fusions are shown in Table 2. Coexpression of Fos and Jun fusions to intact repressor generates heterodimers that block killing by λ phage and efficiently repress transcription from P_R. In contrast, mixed expression of combinations that would form heterodimers with either one arm or no arms have insufficient DNA binding activity to confer superinfection immunity or to efficiently repress P_R–*lacZ*. When cI^+–Fos is coexpressed with (Δ1–6)–Jun, the lack of repressor activity could be due to either the failure of the two inactive fusions to form active heterodimers or the failure of the one-armed heterodimers to bind DNA. However, the reciprocal combination (Δ1–6)–Fos with cI^+–Jun gives a similar efficiency of repression; in this case the armless Fos fusion acts as a dominant negative inhibitor, demonstrating that Fos–Jun heterodimers are forming *in vivo*. Repression by both combinations that form one-armed heterodimers is intermediate between the repression due to the two-armed dimers and the cI^+–Fos fusion alone (Table 1).

Although *lacZ* expression is proportional to the occupancy of O_R *in vivo*, it is not possible to determine from these data the effect of the single and double amputations on the intrinsic affinity of operator binding, since the intracellular concentration of the fusion proteins is not known. If wild-type heterodimers are saturating the operator sites, the relatively small effect on occupancy of amputating an arm could reflect a much larger change in the intrinsic binding affinity. To determine the effect of removing one arm on the intrinsic binding of repressor dimers to operator DNA, we purified Fos and Jun fusion proteins and measured binding *in vitro* by quantitative DNase I footprinting.

To a first approximation, measurements of the DNA binding by different combinations of fusions *in vitro* are consistent with the results observed for repressor activity in *E. coli*. The concentration of protein needed to obtain a given fractional occupancy of O_R1 and O_R3 gives a rank order of Fos–Jun < Jun–Jun ≪ Fos–Fos (Fig. 2). This is consistent with a small difference in stability between Fos–Jun heterodimers and Jun–Jun homodimers, with the former being favored, and both being much more stable than Fos homodimers. None of the fusion proteins binds O_R1 as strongly as the previously observed binding by full-length λ repressor, but all three, including the Fos homodimers, bind better than the N-domain by itself (17). All of the fusions bind O_R2 better than O_R3. This difference is most marked for the Fos homodimers. If the binding was completely noncooperative binding, as is observed for the N-terminal domain alone, binding of O_R2 and O_R3 should occur at comparable protein concentrations (17). These results suggest that there is some cooperativity in operator binding by the fusion proteins. This has not been investigated further.

To measure the effects of removing arms, we compared footprinting by different combinations of Fos and Jun fusion proteins (Fig. 3). We were unable to detect any protection by armless repressor fusion heterodimers at protein concentrations >100-fold higher than that required to give half-maximal binding by heterodimers with two arms (data not shown); this is consistent with the previous work showing that removal of residues 1–6 from λ repressor homodimers reduces operator binding to undetectable levels (4).

To examine binding by one-armed heterodimers, we used a mixture of armless repressor fused to Jun and intact repressor fused to Fos. This combination minimized the binding of the individual components. Removing only one arm from the repressor fusion heterodimer clearly reduces binding to O_R1. In contrast, binding to O_R2 only slightly decreased and binding to O_R3 is relatively unaffected. Moreover, the one-armed repressor has lost most of its ability to discriminate among the three operator subsites.

Because we have not determined the distribution of homodimers and heterodimers in our mixtures, some of the observed binding could be attributed to residual homodimers. Note, however, that protection of O_R3 is observed under conditions where neither the armless Jun fusion nor the intact Fos fusion gives any detectable binding.

DISCUSSION

These results demonstrate that a part of the repressor dimer that is not visible in the well-resolved x-ray structure of the bound complex makes a significant contribution to the stability and specificity of operator binding. How can amino acids 1–6

Table 2. Repression by homodimeric and heterodimeric repressor fusions

tet plasmid		*amp* plasmid				
Repressor domain	Leucine zipper	Repressor domain	Leucine zipper	Arms, no. per dimer	Immunity	% repression
None	—	None	—	None	Sens.	—
None	—	+	GCN4	2	Imm.	86 ± 2
+	Jun	+	Fos	2	Imm.	94 ± 2
Δ1–6	Jun	+	Fos	1	Sens.	51 ± 1
+	Jun	Δ1–6	Fos	1	Sens.	57 ± 3
Δ1–6	Jun	Δ1–6	Fos	0	Sens.	8 ± 3

Compatible plasmids with different antibiotic resistance genes were introduced into JH372 and repression was assayed as described for Table 1. Data for percent repression are the mean ± SEM.

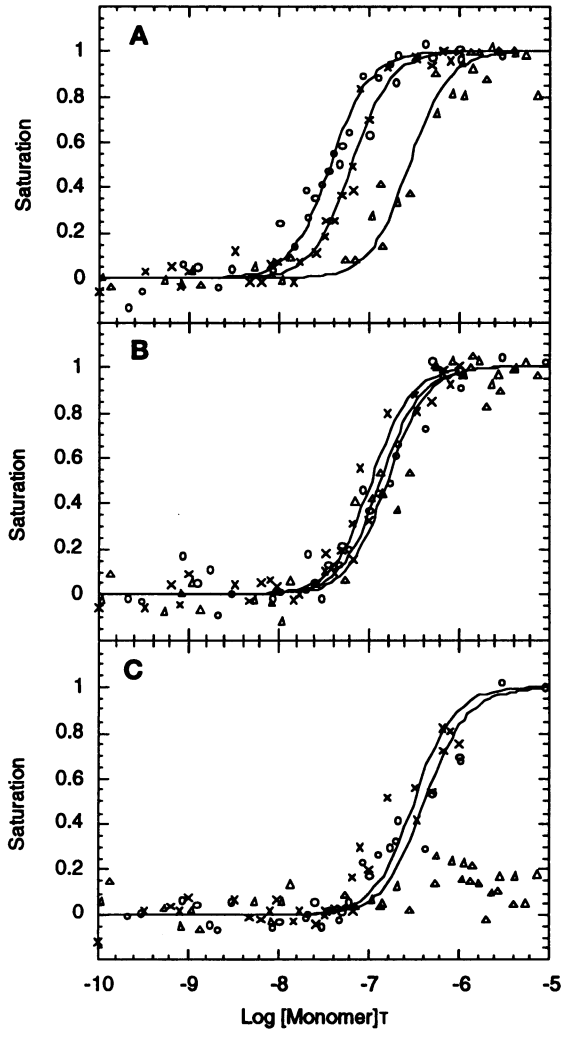

FIG. 2. Effect of the dimerization domain on binding of two-armed fusion proteins to the three operators from O_R. Binding of purified proteins was measured by quantitative DNase I footprinting. (*A–C*) Binding by the N-terminal DNA binding domain of repressor fused to Fos–Jun heterodimers (○), Jun homodimers (x), and Fos homodimers (△). (*A*) Binding to O_R1. (*B*) Binding to O_R2. (*C*) Binding to O_R3. Theoretical binding curves were generated.

of the repressor on the nonconsensus half site contribute to the binding affinity of the dimers without giving visible electron density in the cocrystal structure? Tight-binding λ operators can be divided into consensus and nonconsensus half sites (5). Although both the operator DNA and the repressor dimer are pseudosymmetric, some naturally occurring asymmetric operators bind more tightly to repressor than artificial symmetrized sequences (8). In the cocrystal with O_L1, the visible N-terminal arm lies in the consensus half site. To maintain these contacts, the one-armed repressor fusions should bind with a preferred orientation on λ operators. Since two-armed repressors could bind in either orientation, there should be a 2-fold decrease in the forward rate for the association reaction, even if the nonconsensus arm does not contribute directly to the stability of the complex. However, the observed difference between the one- and two-armed repressors in binding to O_R1 is too large to be accounted for by this model. Instead, we suggest that the nonconsensus arm binds as an ensemble of different structures, each of which would be populated in only a small fraction of the complexes, accounting for the absence of electron density in the cocrystal structure.

The loss of operator affinity due to removing a single arm is clearly much more dramatic for O_R1 than O_R2 or O_R3, such

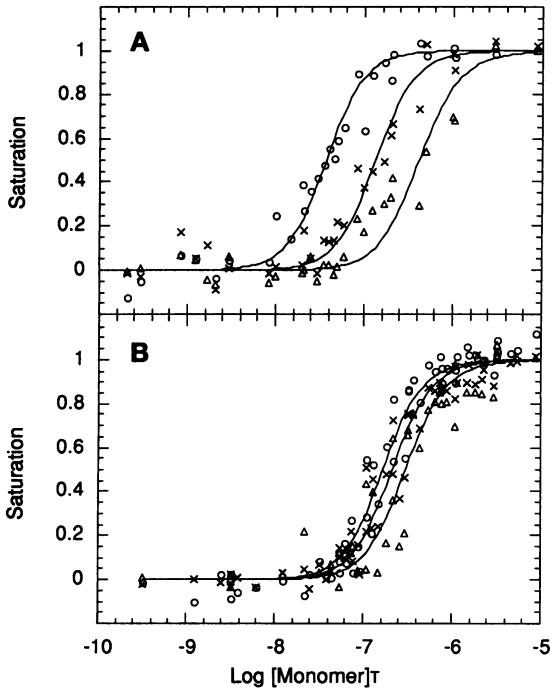

FIG. 3. Binding by two-armed (*A*) and one-armed (*B*) repressor fusions. Fos fused to intact repressor DNA binding domain was mixed with Jun fused to repressor domains with and without residues 1–6. Operator binding was measured by quantitative footprinting on O_R1 (○), O_R2 (x), and O_R3 (△). Theoretical binding curves were generated.

that the one-armed dimer binds the three sites with approximately the same affinity. Eliason *et al.* (4) observed a similar, albeit weaker, loss of specificity when residues 1–3 were removed from both arms of repressor homodimers. Our results suggest that the loss of specificity can be largely attributed to changes in the nonconsensus arm. Five out of seven differences between O_R1 and O_R2 and all of the differences between O_R1 and O_R3 lie in the nonconsensus half site (Fig. 4). The loss of operator discrimination upon removal of one arm suggests that the recognition of differences in the nonconsensus half site has been altered. Although the contact sites for the nonconsensus arm are not known, bases at positions 6′, 4′, 3′, and 2′ of O_L1 make direct contacts with the helix–turn–helix motif of the nonconsensus monomer (6). Of these positions, only position 3′ differs among O_R1, O_R2, and O_R3 (see Fig. 4). Mutations at this position have relatively weak effects (8); the differences in binding by the one-armed repressor fusions due to discrimination of bp 3′ might be too small to observe in our data. Recognition of the other bases by direct contact with the nonconsensus arm might account for the ability of the wild-

	1	2	3	4	5	6	7	8	9	8′	7′	6′	5′	4′	3′	2′	1′
O_R1	T	A	T	C	A	C	C	**G**	C	C	A	G	A	G	G	T	A
	A	T	A	G	T	**G**	**G**	C	**G**	G	T	C	T	C	C	A	T
O_R2	T	A	A	C	A	C	C	**G**	T	G	C	G	T	G	T	T	G
	A	T	T	G	T	**G**	**G**	C	A	C	G	C	A	C	A	A	C
O_R3	T	A	T	C	A	C	C	**G**	C	A	A	G	G	G	A	T	A
	A	T	A	G	T	**G**	**G**	C	**G**	T	T	C	C	C	T	A	T

FIG. 4. Three operator sites in O_R arranged so that their consensus half sites are aligned. Base pairs that are conserved in all six λ operators are boxed. Base pairs that differ from consensus are shown in outline type. Bases in the consensus half site that interact with residues in the N-terminal arm (5, 6) are shown in boldface type.

type repressor to distinguish among the three operator sites in O_R.

Alternatively, loss of the nonconsensus arm could indirectly affect the interaction of the helix–turn–helix motif with the operator in at least two classes of models. In the first model, the removal of the nonconsensus arm would relieve a steric clash between the arms in the center of the operator proposed by Sarai and Takeda (8) to explain the asymmetry of repressor binding to operator DNA. This would allow the helix–turn–helix motif bound to the nonconsensus half site to reorient in the major groove to make contacts more similar to those found between the consensus half site and the helix–turn–helix motif of the other monomer. By assuming that substitutions in the nonconsensus half site would now have energetic effects comparable to equivalent positions in the consensus half site (8), the differences in binding among the three operators would be dramatically reduced (Y. Takeda, personal communication).

In the second model, the loss of specificity of the one-armed heterodimers could reflect different binding modes for strong and weak operators. Base-specific or backbone contacts made by the nonconsensus arm could be required to orient the helix–turn–helix motif on strong binding sites like O_R1 and O_L1. The arms could be envisioned as clamping down the DNA binding domains so that the helix–turn–helix motifs are pulled into the major groove of the DNA. In the complex between the one-armed repressor and O_R1, only the subunit bound to the consensus site would be properly seated into the DNA. The subunit bound to the nonconsensus site would be oriented differently relative to the DNA, leading to either a partial or complete loss of specificity. If binding of both the wild-type and the one-armed repressor to O_R3 occurred with the nonconsensus subunit in this alternative orientation, then the removal of one arm would have a smaller effect on binding. The possibility that repressor dimers bind O_R1 and O_R3 in different conformations is consistent with spectroscopic studies comparing complexes with repressor bound to O_L1 and O_R3 (21) and the observation of a similar loss of operator specificity in mutations that affect the packing of the interface between the two N-terminal domains (D. Senear, personal communication). The models proposed above make different predictions about how the one-armed repressor will behave on other mutant operator sequences.

Our finding that the one-armed repressor changes both the affinity and specificity of operator DNA binding raises questions about how to interpret the effects of substitution mutants in the N-terminal arm. Clarke *et al.* (5) have shown that within residues 1–6, only residues 3, 4, and 5 lose DNA binding activity when replaced with nonconserved amino acids. These sequence constraints can be understood in terms of the structure of the visible arm in the cocrystal. However, the invisible arm must also contribute to the amino acid sequence requirements for the arm. For example, in the crystal structure Lys^4 is the only amino acid that contacts bp 7 in the consensus half site (5, 6). To rationalize the effects of operator mutations at that position (8), this contact must be energetically significant. However, the effects of substitution mutations at Lys^4 (5) might also reflect the requirement for Lys^4 in the arm associated with the nonconsensus half site. An analysis of the effects of other substitution mutants in the N-terminal domain in the context of heterodimeric fusion proteins should provide some insights into how repressor binds operator DNA.

We thank T. Baldwin, J. Eliason, D. Giedroc, F. Gimble, C. Gross, R. Sauer, D. Siegele, D. Shippen, Y. Takeda, and X.-G. Zeng for reading various versions of this manuscript and providing many insightful comments. We thank D. Senear for generously sharing unpublished results. We thank E. Nicholson and M. Scholtz for help with circular dichroism measurements and L. Guarino for the use of the slot blotting apparatus for filter binding. The FUJIX imaging system used at the Gene Technologies Laboratory was purchased with funds from National Science Foundation Grant BIR9217251. This work was supported by funding from National Science Foundation Grant MCB-9305403 to J.C.H.

1. Pabo, C. O. & Sauer, R. T. (1992) *Annu. Rev. Biochem.* **61,** 1053–1095.
2. Weiss, M. A., Sauer, R. T., Patel, D. J. & Karplus, M. (1984) *Biochemistry* **23,** 5090–5095.
3. Pabo, C. O., Krovatin, W., Jeffrey, A. & Sauer, R. T. (1982) *Nature (London)* **298,** 441–443.
4. Eliason, J. L., Weiss, M. A. & Ptashne, M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2339–2343.
5. Clarke, N. D., Beamer, L. J., Goldberg, H. R., Berkower, C. & Pabo, C. O. (1991) *Science* **254,** 267–270.
6. Beamer, L. & Pabo, C. (1992) *J. Mol. Biol.* **227,** 177–196.
7. Pabo, C. O., Sauer, R. T., Sturtevant, J. M. & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1608–1612.
8. Sarai, A. & Takeda, Y. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6513–6517.
9. Hu, J., Newell, N., Tidor, B. & Sauer, R. (1993) *Protein Sci.* **2,** 1072–1084.
10. Hu, J. C., O'Shea, E. K., Kim, P. S. & Sauer, R. T. (1990) *Science* **250,** 1400–1403.
11. Reidhaar-Olson, J., Bowie, J. U., Breyer, R. M., Hu, J. C., Knight, K. L., Lim, W. A., Mossing, M. C., Parsell, D. A., Shoemaker, K. R. & Sauer, R. T. (1991) *Methods Enzymol.* **208,** 564–586.
12. Arber, W., Enquist, L., Hohn, B., Murray, N. E. & Murray, K. (1983) in *Lambda II,* eds. Hendrix, R., Roberts, J., Stahl, F. & Weisberg, R. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 433–466.
13. Meyer, B. J., Maurer, R. & Ptashne, M. (1980) *J. Mol. Biol.* **139,** 163–194.
14. Miller, J. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
15. Riggs, A., Suzuki, H. & Bourgeois, S. (1970) *J. Mol. Biol.* **48,** 67–83.
16. Brenowitz, M., Senear, D., Shea, M. & Ackers, G. (1986) *Methods Enzymol.* **130,** 132–181.
17. Johnson, A. D., Meyer, B. J. & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5061–5065.
18. O'Shea, E. K., Rutkowski, R., Stafford, W., III, & Kim, P. S. (1989) *Science* **245,** 646–648.
19. Blattner, F. R., Fiandt, M., Hass, K. K., Twose, P. A. & Szybalski, W. (1974) *Virology* **62,** 458–471.
20. Meyer, B. J., Maurer, R. & Ptashne, M. (1980) *J. Mol. Biol.* **139,** 163–194.
21. Benevides, J., Weiss, M. & Thomas, G. J. (1991) *Biochemistry* **30,** 5955–5963.