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A repressor heterodimer binds to a chimeric operator

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ABSTRACT **Replacement of the solvent-exposed residues of the DNA recognition helix of the 434 repressor with the corresponding residues of the P22 repressor generates a hybrid protein, 434R[α3(P22R)], which binds specifically to P22 operators. We show here that a new DNA-binding specificity is generated by combining 434 and 434R[α3(P22R)] repressor monomers to form a heterodimer. The heterodimer specifically recognizes a chimeric P22/434 operator that lacks two-fold rotational symmetry.**

The bacteriophage 434 repressor recognizes its operator DNA as shown in part in Fig. 1. The repressor binds as a dimer, inserting two identical α-helices (recognition helices) into successive major grooves along one face of the DNA. Each recognition helix lies in one-half of the operator, and amino acids on the outside surface of the helix make specific contacts with functional groups exposed in the major groove of the DNA (1). The DNA-binding form of the repressor is a dimer, and dimerization is mediated primarily by the carboxyl domain; the recognition helix is found in the amino domain (2). In the protein–DNA complex, the axis of two-fold symmetry of the protein is coincident with that of the two-fold symmetric operator (1).

A number of proteins, including the repressors of coliphages λ and 434 and the *Salmonella* phage P22 repressor, use a recognition helix to recognize their operator DNA (see ref. 3 for review). In the "helix-swap" experiment of Wharton and Ptashne (4), the solvent-exposed residues of the 434 repressor recognition helix (referred to as α3 since it is the third α-helix in the protein) were replaced with the corresponding residues from the recognition helix of the P22 repressor. The resulting protein {434R[α3(P22R)]} bound specifically and with high affinity to P22 operators. Except for four amino acids on the outside face of the recognition helix, 434R[α3(P22R)] is identical to 434 repressor.

In this paper we show that monomers of 434 and 434R[α3(P22R)] repressors form mixed dimers that recognize a hybrid, nonsymmetric 434/P22 operator with high affinity.

MATERIALS AND METHODS

Materials. All enzymes were purchased from New England Biolabs with the exception of DNase I, which was purchased from Miles Laboratories. Oligonucleotides were synthesized by the Harvard Microchemistry Facility or by M. Edge (Imperial Chemical Industries).

Protein Purification. 434R[α3(P22R)] repressor was purified from cells carrying plasmid pRW219 as described (4). 434 repressor, purified by the method of Anderson *et al.* (5), was a kind gift of G. Koudelka (Harvard University). Both proteins were assayed for activity and found to be >85% active (R.W., unpublished data; G. Koudelka, personal communication).

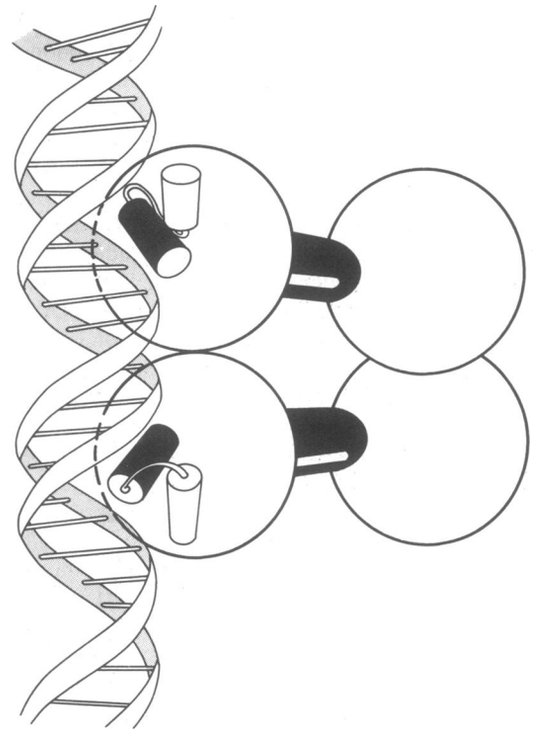

FIG. 1. Schematic diagram of 434 repressor bound to DNA. A dimer of 434 repressor bound to its 14-base-pair (bp) operator is shown. Each monomer is shown as two domains, connected by a linker region. The carboxyl-terminal domains, which mediate dimerization, are shown away from the DNA, whereas the amino-terminal domains contact the operator. The conserved helix-turn-helix motif in each monomer is indicated as a pair of cylinders. The recognition helix, which makes specific contacts to residues in the major groove of the DNA, is shown in black.

Cloning of Hybrid Operators. Double-stranded synthetic oligonucleotides carrying the hybrid operators flanked by *Sal* I-compatible ends were cloned into the *Sal* I site of plasmid pUC18 (6) to generate plasmids pAD15, pAD16, pAD17, pMUT1, and pMUT3. Plasmid pMUT2 was generated by mismatched primer mutagenesis as outlined below. The polylinker region of plasmid pAD16 was recloned into plasmid pEMBL8+ (7) to give pDV50. Preparation of single-stranded pDV50 DNA from *Escherichia coli* strain RZ1032 (ATCC 39737; ref. 8) and subsequent mutagenesis were as described by Zoller and Smith (9). All operator sequences were verified by plasmid sequencing using the method of Chen and Seeburg (10).

Filter Binding. The hybrid operators were excised from the corresponding plasmids as ≈80-bp *Eco*RI–*Hin*dIII fragments and were 5′ end-labeled to high specific activity at either the *Eco*RI or *Hin*dIII end using polynucleotide kinase and

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[^{32}P]dATP as described (11). Nitrocellulose filter binding assays (12) were as described below. The labeled operator fragments were incubated with the repressors at a final concentration of <20 pM in 400 μl of 50 mM sodium cacodylate, pH 8/10 mM magnesium chloride/0.1 mM disodium EDTA/50 mM potassium chloride (CB buffer) containing 5 μg of sonicated chicken blood DNA per ml and 50 μg of bovine serum albumin per ml. 434 and 434R[α3(P22R)] repressors were diluted on ice to a concentration of 1 μM in CB buffer (plus bovine serum albumin) and aliquots of the diluted proteins were mixed in equal proportions to give the "heterodimer" sample. The three diluted protein samples were immediately added to parallel binding reactions to give a final range of repressor concentrations from 1.5 nM to 100 nM. After incubation at room temperature for 15 min, the samples were filtered through 24-mm nitrocellulose filters (Millipore, type HA, 0.45 μm) that had been presoaked in CB buffer. The retention of labeled operator DNA was measured by Cerenkov counting of the filters. A sample without repressor was included to give a background retention (always <6%), which was subtracted from the measured values.

DNase I Protection Experiments. DNase I protection experiments were performed essentially as described by Johnson *et al.* (13) except that CB buffer containing 5 μg of sonicated chicken blood DNA per ml and 50 μg of bovine serum albumin per ml was used for repressor binding and DNase I cleavage reactions. DNA fragments containing the hybrid operators were prepared and labeled as described above for filter binding.

RESULTS AND DISCUSSION

Experimental Plan. The important molecules used in this study are diagramed in Fig. 2. 434 and 434R[α3(P22R)] repressor dimers are shown bound to their cognate operators. The heterodimer is shown bound to a hybrid operator, a prediction we confirm below. Because the repressors differ only along the outside of their respective recognition helices (as diagramed in Fig. 2), they share all of the sequences necessary for dimerization and should efficiently form the indicated heterodimer. The heterodimer should recognize a hybrid operator, reflecting the contribution of each monomer to the binding specificity.

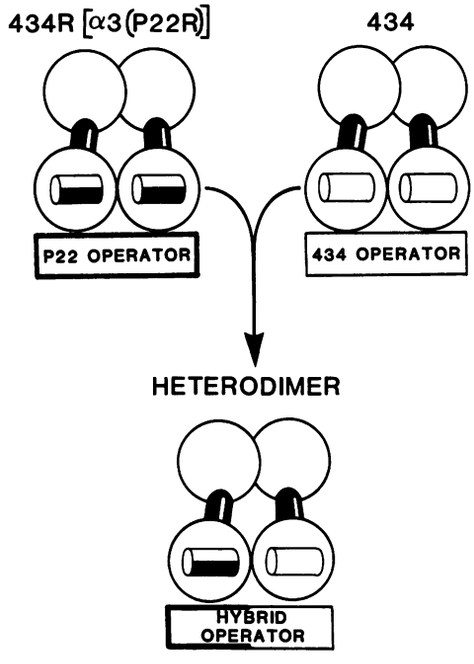

FIG. 2. Plan of experiment. 434R[α3(P22R)] and 434 repressor dimers are schematically shown bound to their corresponding operators. In each case, the recognition helix of each monomer is shown as a cylinder. 434R[α3(P22R)] is identical to 434 repressor except that it bears the solvent-exposed residues of the P22 recognition helix (shown black) and consequently binds to P22 operators as indicated. After mixing, a heterodimer of the two proteins is shown bound to a hybrid P22/434 operator, where each repressor monomer is bound to its cognate half-site.

Fig. 3*A* shows the consensus operator sequences recognized by the 434 and P22 repressors. The 434 and P22 operators are 14 and 18 bp, respectively (14, 15), each with a center of symmetry between the central base pairs. In each case, the outer base pairs of the operators are most highly conserved. For the 434 case, crystallography (1) and biochemical mutagenesis (4, 14, 16) show that these base pairs are contacted by side chains of the amino acids of the recognition helix, and we presume the same to be true in the P22 case.

To test the accuracy of our prediction, we measured the affinity of the three hybrid operators shown in Fig. 3*B*. One of these sites (the 16-bp hybrid operator) corresponds to a 9-bp P22 operator half-site fused to a 7-bp 434 half-site. The other sites differ from the 16-bp operator in that they contain either an insertion (17-bp site) or a deletion (15-bp site) of a single base at the center of the operator. We measured, using filter binding, the affinity of these operators for 434 repressor, 434R[α3(P22R)] repressor, and a mixture of the two repressors.

Filter Binding. Fig. 4 shows that 434 and 434R[α3(P22R)] repressors bind only with barely detectable affinity to the hybrid sites. In contrast, a mixture of 434 repressor and 434R[α3(P22R)] repressor bound the 16-bp hybrid operator with high affinity. From Fig. 4 the dissociation constant for this interaction is 5 nM. In a parallel experiment, performed under identical conditions, the dissociation constant of 434 repressor for its strong binding site O_R1 was 2 nM (data not shown).

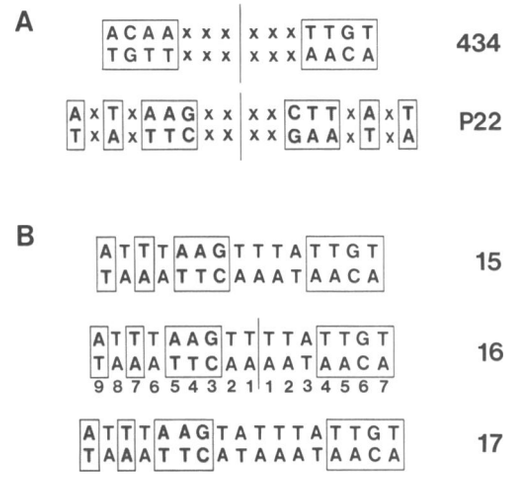

FIG. 3. Operator sequences. (*A*) Consensus operator sequences for 434 and P22 repressors (14, 15). A vertical line marks the center of symmetry of each sequence. The conserved bases in each operator are boxed, and nonconserved bases are indicated by an X. (*B*) Sequences of the three hybrid P22/434 operators used in this study. The conserved bases of the P22 and 434 half-sites are boxed as in *A* above (stippled boxes for P22; open boxes for 434). The lengths of the operators, defined as the distances between the outer conserved bases in each half-site, are indicated. The 16-bp hybrid operator consists of a consensus P22 half-site fused to a consensus 434 half-site; the fusion junction is indicated by a vertical line. The three hybrid operators differ in the spacing between the conserved bases of each half-site (as discussed in the text).

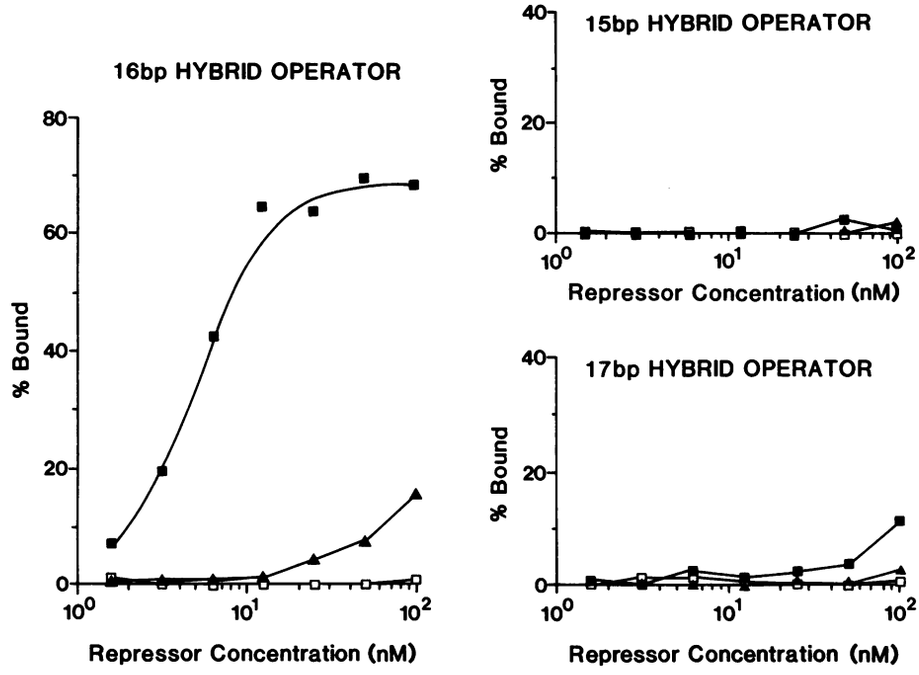

FIG. 4. Repressor binding to the hybrid operators. Each of the hybrid operators described in Fig. 3 was assayed by filter binding with 434 repressor (□), 434R[α3(P22R)] repressor (▲), and a mixture of the two repressors (■) over the concentration range indicated. For the mixed repressor sample, the concentration of heterodimers was taken to be 50% of the total {434 + 434R[α3(P22R)]} repressor protein in solution.

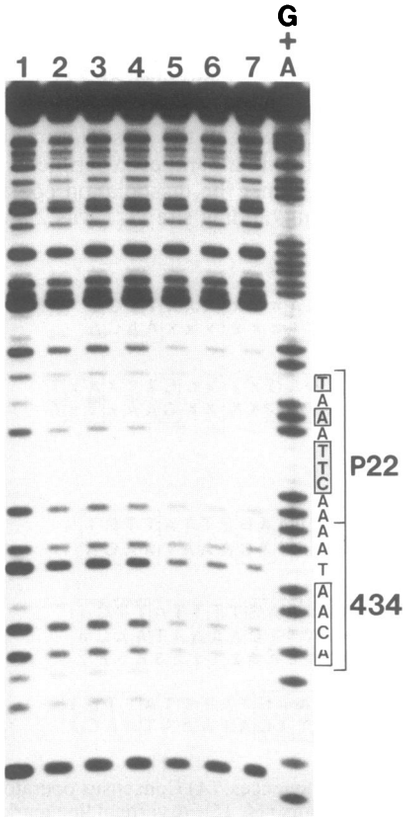

FIG. 5. DNase I protection of the 16-bp hybrid operator. An *Eco*RI–*Hin*dIII fragment of plasmid pAD16, which carries the hybrid operator, was 5′ end-labeled at the *Hin*dIII site and incubated with serial dilutions of the mixed repressor preparation. The heterodimer concentrations in each reaction mixture were as follows: lane 1, no repressor; lane 2, 1.6 nM; lane 3, 3.1 nM; lane 4, 6.2 nM; lane 5, 12.5 nM; lane 6, 50 nM; lane 7, 100 nM. The lane marked G + A is the same DNA fragment cleaved at purines as described (11). The sequence of the hybrid operator is shown as in Fig. 3, and the positions of the 434 and P22 half-sites on the fragment are marked.

Why does the 16-bp operator bind with high efficiency, whereas the 15-bp and 17-bp operators do not? We imagine that the center of symmetry of the heterodimer is centered on the junction between the operator half-sites indicated in Fig. 3*B*, line 2. In this case the distance to the contacted base pair (T·A) at the outside of the 434 half-site is the same as that to the T·A base pair at position 7 (from the junction) in the P22 half-site. If we assume that the outer A·T base pair of the P22 half-site (position 9) is not contacted (there is no firm evidence on this point), then the distance from the center to the outer contacted base in each half-site is the same. Moreover, this distance is the same as that separating the

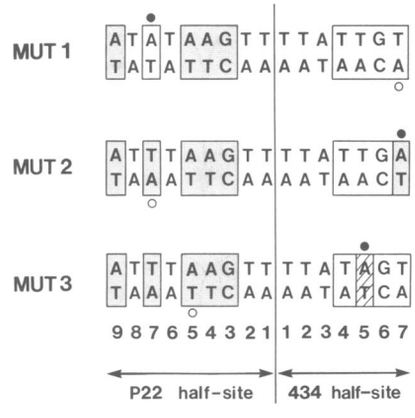

FIG. 6. Sequences of mutant operators. The sequences of three mutant 16-bp hybrid operators are shown. The sequences have been divided into P22 and 434 half-sites, and the junction between half-sites is indicated with a vertical line. The bases in each half-site are numbered from the junction. Each mutant operator carries a single base substitution (marked ●) with respect to the 16-bp hybrid operator shown in Fig. 3*B*; for MUT1, T·A → A·T at position 7 in the P22 half-site; for MUT2, T·A → A·T at position 7 in the 434 half-site; and for MUT3, T·A → A·T at position 5 in the 434 half-site. The conserved residues in each half-site are boxed as in Fig. 3. Bases that are symmetrically equivalent to the mutant bases (●) are marked with open circles (○).

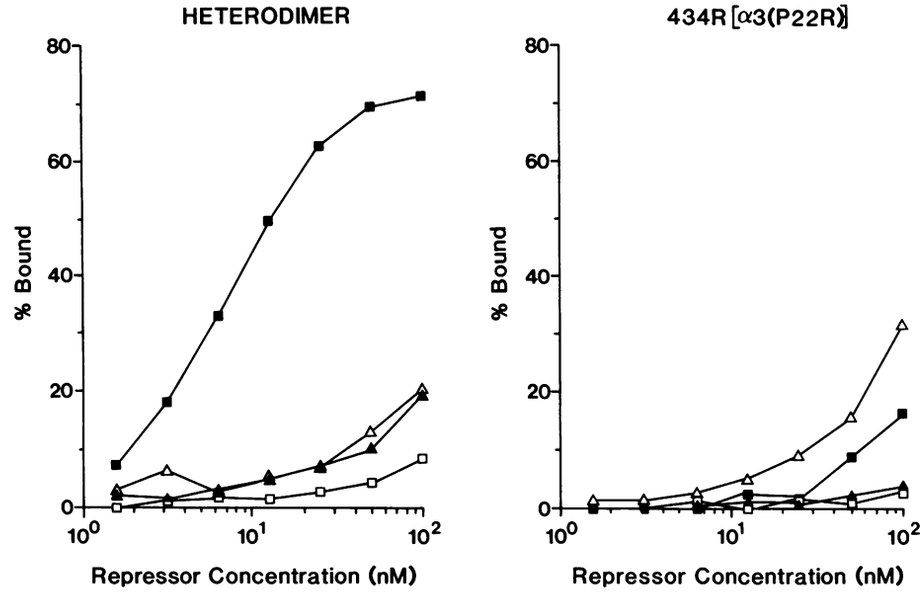

FIG. 7. Repressor binding to the mutant operators. 434 repressor, 434R[α3(P22R)] repressor, and a mixture of the two repressors (marked HETERODIMER) were assayed for binding to the mutant operator sites. In each case, the binding of the mutant sites MUT1 (□), MUT2 (△), and MUT3 (▲) was compared directly to that of the 16-bp hybrid operator site (■). For the 434 repressor, no detectable binding was seen with any of the hybrid operators tested (data not shown).

center of symmetry and the outer contacted bases in the natural 434 operators. In other words, according to this idea, the 434R[α3(P22R)] and 434 recognition helices are each positioned on the 16-bp hybrid operator just as the recognition helices of 434 repressor are on the wild-type 434 operator. In the other two hybrid operators it is not possible to draw a center of symmetry so that this feature would hold.

DNase I Protection. Fig. 5 shows the results of DNase I protection experiments with the repressor mixture and the 16-bp hybrid site. The results show that the repressor mixture recognizes the operator specifically as expected. The size of the protected region (≈25 bp) corresponds to that seen for the binding of a single 434 or 434R[α3(P22R)] repressor dimer to a single binding site (4, 14). Under the same conditions, no "footprint" was seen for either 434 or 434R[α3(P22R)] repressor alone (data not shown).

Mutant Operators. The experiments described above indicate that the hybrid operator is recognized by a heterodimer containing one monomer of 434 repressor and one monomer of 434R[α3(P22R)] repressor. We imagine that the 434 recognition helix makes specific contacts in one half-site of the hybrid operator and the recognition helix of P22 repressor {in 434R[α3(P22R)]} contacts the other. To further test this idea, we synthesized three mutant operators (shown in Fig. 6). Two of the mutants bear changes in the 434 half-site of the hybrid operator. One of the changes (in MUT2) makes that half-site more closely resemble a P22 operator half-site, whereas the other (MUT3) makes that half-site less closely resemble either a P22 or a 434 half-site. The third mutant (MUT1) bears a change in the P22 half-site that makes that half-site more closely resemble a 434 half-site. Two of the changes (in MUT1 and MUT2) make the hybrid operator more symmetric than the parent 16-bp hybrid operator.

Fig. 7 shows that each of these changes strongly decreases the affinity of the operator for the repressor mixture. This is exactly the result expected on the assumption that the active species is a heterodimer that recognizes an operator bearing one intact 434 half-site and one intact P22 operator half-site. The mutant operator, which more closely resembles a P22 operator (MUT2), does in fact have a slightly increased affinity for the 434R[α3(P22R)] repressor.

The experiments presented here show that two DNA-binding proteins can be combined to form another species that recognizes a new DNA sequence. The new operator sequence is a hybrid of the natural operators but, unlike those operators, has no two-fold rotational symmetry. Might nature use this principle to extend the number of sequences recognized by regulatory proteins? In several cases it has been proposed that regulatory proteins recognize specific asymmetric sequences as heterodimers (17–20). Our results provide an example of how this can be achieved.

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